CPS CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Strategic approaches to mitigate *Salmonella* contamination of bulb onions

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Objectives

1. *Understand the impact of transcriptomic and metabolomic factors on Salmonella colonization in onion varieties.*
2. *Identify and evaluate the impact of pre- and post-harvest determinants involved in onion production practices on Salmonella contamination.*
3. *Characterize the establishment and internalization of Salmonella in onion.*

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FINAL REPORT

Abstract

After exposure to *Salmonella*, the transcriptomic analysis of onion bulbs confirmed the differential expression of genes associated with plant-pathogen interactions, suggesting the role of plant innate immunity. Similarly, the induction of genes related to *Salmonella*'s Type III secretion system indicates that the pathogen tries to counteract the plant immune responses and activating mechanisms to colonize onion bulb scales. The chemical analysis confirmed the activation of several defense-related metabolites, particularly antibacterial compounds like quercetin and its derivatives in onion bulbs after inoculation with *Salmonella*. To determine the survival of internalized and non-internalized *Salmonella* in onions, a challenge study was conducted, where *Salmonella* ser. Newport was inoculated at various depths in and on red, white, and yellow onions and then stored at room temperature for up to 18 days. At intervals, samples were collected from each onion's top layer (surface) and inner layers and tested for *Salmonella* numbers. In all cases, the outer layer of all onion varieties not only inhibited *S*. Newport growth, but the populations of this pathogen were reduced by 3.2, 2.4, and 2.5 log cycles on red, white, and yellow onions, respectively, within the first 3 days of storage, with no significant further changes in counts over 18 days. In contrast, S. Newport grew by 1.7, 2.3, and 2.5 log cycles over the 18-d storage time in the inner layers of red, white, and yellow onions. In the trials to track the presence of *Salmonella* in onion-producing environments, samples were collected from onion fields and onion packing plants in the Winter Garden area of Texas. A total of 255 samples (101 from fields and 154 from packing plants) were collected. Field samples consisted of water and soil, whereas packing plant samples were collected from various surfaces in the plant environment. No food-contact surface samples were collected. All samples were subjected to qualitative and quantitative *Salmonella* assay in a BAX system with the SalQuant kit. Samples that tested positive were confirmed with cultural methods. *Salmonella* was detected in 3 (13%) of 23 samples of well water collected from one onion field, 3 (4%) of 75 samples of soil collected from three fields, and 16 (10%) of 151 surface samples collected from three onion packing plants. In positive field samples, the quantitative assay gave mean counts of 0.1 log CFU/50 L in water and 1.2 log CFU/g in soil. For environmental samples from packing plants, the mean counts for positive samples ranged between 1.9 and 3.2 CFU/cm^2; no differences between types of surfaces were found in the *Salmonella* counts ($P > 0.05$).

Background

This project aimed to address a multistate outbreak of *Salmonella* Newport, resulting in recalls by the Food and Drug Administration (FDA) of multiple onion varieties and products containing onions in early May 2020. As the number of outbreaks and *Salmonella* cases continues to escalate in other agricultural produce, such incidences in onion production and supply chain have also risen, including the imported produce. These outbreaks warrant a cautious approach to avoid or eliminate future *Salmonella* contamination in onions. While the FDA continues with its root cause investigation, we proposed a systematic approach to curb future outbreaks of *Salmonella* in dry bulb onion based on a scientific study. Despite the potential risk of similar outbreaks, currently, we do not have a strategic plan to prevent *Salmonella* contamination in onions due to two bottlenecks: 1) lack of knowledge about the mode of colonization and internalization of *Salmonella* in onions, and 2) limited understanding of the determinants that would impact the onion-specific production practices for ensuring safe produce. The objectives of this 2-year project are: (1) To understand the impact of transcriptomic and metabolomic factors on *Salmonella* colonization in onion varieties, (2) To identify and evaluate the impact of pre-and postharvest determinants involved in onion production practices on *Salmonella* contamination, and (3) To characterize the establishment and internalization of *Salmonella* in onion. A parallel workflow will be established to meet these three objectives, where onion-specific component responses induced due to *Salmonella* colonization will be evaluated using transcriptomic and metabolomic analyses (Objective 1), along with the simultaneous evaluation of the *Salmonella* attachment and allocation on onion structures (Objective 3). To better understand the role of pre-and postharvest bulb onion production practices on the susceptibility to *Salmonella* contamination, a multifactorial field study will evaluate the role of bulb nutrient and quality traits defined by the genotype, environment, and management practices. Through the onion supply chain interventions, samplings will be conducted at the onion production sites or packing sheds to monitor the conduciveness of the environment for *Salmonella* establishment. This study will identify differential transcriptomic and metabolic responses to identify molecular/metabolic markers and establish strategies for breeding Salmonella-safe onion varieties. An in-depth analysis of *Salmonella* internalization on onion bulb sheath and its stability would allow other researchers to engage in developing technologies that would eliminate or irradiate possibilities of future outbreaks in onion. Further, by creating a roadmap for safe production practices, it will be possible to model *Salmonella*'s presence in the supply chain based on production practices, seasonal variation, and varieties and minimize the risk of pre/postharvest contamination in onion fields.

Research Methods and Results

Objective #1. Understand the impact of transcriptomic and metabolomic factors on *Salmonella* colonization in onion varieties.

Transcriptomic analysis. Duel transcriptomic analysis of onion bulbs inoculated with *Salmonella* strain was performed 24 hours and 7 days post-inoculation (See image below). The *Salmonella* cocktail strains of *Salmonella enterica* subsp. *enterica* ser. Newport, isolated from clinical cases affected by the onion-linked outbreak in 2020 (CDC, 2020), was used for the onion bulb inoculation. All outbreak-related *Salmonella* Newport strains, CC1070, CC1071, CC1072, and CC1073, were obtained from David Mann (University of Georgia - Center of Food Safety; additional details under objective 3).

Transcriptomics. Total RNA was extracted from each sample using the QIAseq fastSelect rRNA kits. The mRNA was isolated from the total RNA using magnetic beads with Oligo (dT) and fragmented into short segments using a fragmentation buffer. cDNA was synthesized via reverse transcription (Superscript III, Invitrogen). The ends of the double-stranded cDNAs were then repaired and tailed by the Klenow enzyme, and universal TruSeq adapters (Illumina) were ligated. The DNBSEQ platform by BGI Tech was used to construct RNA-seq libraries for onion and Salmonella samples (each with three biological replicates). The raw sequencing data will be uploaded to the NCBI BioProject database along with the publication (under preparation). The onion RNA-seq clean data were mapped onto the full-length transcript sequences acquired from de novo assembly and ab initio annotation of the genome of a doubled haploid onion line DHCU066619 (https://www.oniongenome.wur.nl/) and Salmonella transcriptomic reads was mapped to GenBank assembly GCA_027235085.1 sequenced by FDA Center for Food Safety and Applied Nutrition.

Bioinformatic analysis. The read count for every transcript was determined from the findings of the mapping process. The expression levels were calculated and normalized to Fragments Per Kilobase of transcript sequence per million base pairs sequenced (FPKM) using RSEM (Li & Dewey, 2011). The DESeq2 R package (1.20.0) was used for the differential expression analysis. The P-values obtained were adjusted using Benjamini and Hochberg's method to control the false discovery rate. Genes identified as differentially expressed genes (DEGs) had an adjusted P-value <0.05 and an absolute log2FC ≥0, as determined by DESeq2. The cluster Profiler R package was utilized to carry out GO and KEGG pathway enrichment analyses of the DEGs.

Metabolite analysis. Widely targeted metabolomics of inoculated onion bulbs was performed using three onion varieties (red, white, and yellow). 36 samples (including control and inoculated bulbs) were selected and divided into 12 groups for metabolic study, with 3 biological replicates for each group. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used for the metabolomics analysis to detect and identify metabolites with important biological significance by differentiating statistically significant differential metabolites between sample groups. Lyophilized samples were homogenized using a ball mill grinder, and 50 mg ground sample was extracted with 70% methanol with internal standards. After centrifugation, the filtered extracts (0.22 um) were used for the UPLC-MS/MS analysis. The data acquisition was performed using UPLC (Ex- ionLC™ AD, https://sciex.com/) and tandem mass spectrometry (MS/MS) (Applied Biosystems QTRAP 6500). The metabolites were identified qualitatively based on their secondary spectrum information. Metabolites were quantified by triple quadrupole mass spectrometry with multiple reaction monitoring (MRM). The standard statistical analysis was performed using JMP software or R scripts as appropriate.

Objective #2. Identify and evaluate the impact of pre- and postharvest determinants involved in onion production practices on *Salmonella* contamination.

Onion-replicated field experiments were conducted at the Texas A&M AgriLife Research Center in Uvalde, Texas. Regionally popular onion varieties (total=12; 3 red, 3 yellow, 3 white, and 3 sweet yellow) were used for the field study. Varieties were exposed at two nitrogen fertilization levels (150 lb/acre considered HN, and 75 lb/acre as deficit LN) and two water levels based on crop evapotranspiration rates, ETc (100% ETc considered well-watered HW, and 60% ETc as water deficit, LW). The split-plot design with four replications per treatment was used, with nitrogen-water levels as the main plot factor and varieties as subplots. Seeds were planted with a vacuum planter on single raised beds separated 40" apart with four lines per bed and seed spacing of 3.75". Pre-plant fertilization was incorporated into beds before transplanting with

50N-0P-50K lb/acre. Additional post-plant fertilization was provided as fertigation through the subsurface drip system based on pre-plant soil nutrient analysis. Standard practices used by onion growers in the region include weed, pest, and disease control. At maturity, bulbs were sorted mechanically by diameter, and representative samples of 15 onion bulbs per treatment/replication were used for further analysis.

Onion sourcing and extract preparation. Carta Blanca (white), Red Label (red), and Dulciana (yellow) onions were grown in controlled soil conditions consisting of nominal water, referenced (66 cm) or an imposed stress of lower water (46 cm) and optimal nitrogen (0.013 kg/m2) or sub-optimal (0.007 kg/m2). Extracts from these onions were made using lyophilization and methanol extraction following a modified protocol from Sagar and Pareek (2020). Onion extract concentrations of total phenolic compounds were determined by spectrophotometric analysis using gallic acid as a standard, and flavonoid concentration was determined by spectrophotometric analysis using quercetin as a standard. Anthocyanins were also reported. These bioactive compounds will be combined as all the compounds were considered to contribute an effect on bacterial inhibition. As their impact cannot be extrapolated individually, their combined concentration was used to calculate minimum inhibitory and bactericidal concentrations, referred to as total active compounds (TOC). To study the effect of growing conditions and genotype on the inhibition capacity of onion extracts, all procedures followed for these trials were the same as described for MIC and MBC (detailed under Objective 3), except that the concentration of TOC was not determined. The inhibitory effect was therefore studied on the lowest dilution (highest concentration) of the extracts.

Inhibition of *Salmonella* by onion extracts made from different genotypes of onion. Elite parental lines from Texas A&M Breeding Germplasm were used to evaluate the impact of genotype on the *Salmonella* establishment. Onions corresponding to genotypes 218Y, 541X, 1196, Yellow H6, 545X, 25X, 542, 1197, 1102, 231Y, 544X, and 1104 were grown at the Uvalde AgriLife Center, harvested and used to prepare extracts as described above. For all 12 genotypes, the extracts were prepared from inner and outer scales to determine the effect of the potential concentration of active compounds. The effect of the lowest dilution of the extract (1:2) was used for regression studies to determine the effect of the genotype on the inhibitory activity of the onion extracts. Additionally, to understand the impact of the type of lights on the onion bulbs during storage, onion cultivars Sofire (red), White (white), and Rio Dulciana (yellow) were grown in a greenhouse under red, blue, or white light. After harvest, extracts were prepared and sent to the microbiology laboratory, where inhibition trials were conducted following the microplate assay method described above. Again, the lowest dilution was used to study the extracts' inhibitory or bactericidal effect.

Objective #3. To characterize the establishment and internalization of *Salmonella* in onion.

Test the effect of onion components on the survival of *Salmonella.* Inhibitory effect of onion extracts against *Salmonella:* A cocktail of 5 *Salmonella enterica* subsp. *enterica* strains, each corresponding to serovars Enteritidis, Michigan, Poona, Saint Paul and Typhimurium, was used as inoculum for the challenge studies using onion extracts. Each isolate was grown overnight on Muller-Hinton Broth (Difco, Sparks, MD) and then washed 3 times by centrifugation, resuspending in phosphate-buffered saline (PBS, Calbiochem). Then, suspensions of each washed culture were prepared to an equivalent of a McFarland 0.5 standard (~8.2 log CFU/ml) (bioMerieux), using sterile isotonic saline solution and then diluted in PBS to a target inoculum level of 5.7 log CFU per well in a 96-well sterile microplate. The actual concentration in the inoculum was verified by spread plating on tryptic soy agar (TSA, Difco).

Onion extracts screening using 6mm filter discs. Lyophilized onion extracts were reconstituted in 10 ml sterile distilled water and filtered using a 0.2 um filter. A preliminary test of the inhibitory effect of onion extracts was conducted using the agar diffusion method. Muller-Hinton agar plates were inoculated with the *Salmonella* cocktail described above. The bacterial concentration of the cocktail was determined to be 8.0 log CFU/ml by plate counts. Muller-Hinton agar plates were inoculated using cotton swabs, and then sterile filter discs (6mm diameter) were placed on the surface of the inoculated agar and impregnated with 50 µl of onion extract or control. Ampicillin sodium (128 µg/ml) was used as a positive control. Each assay was carried out in triplicate. Petri dishes were incubated at 37°C for 24 hours, and inhibition zones were measured using a caliper.

Microplate assay for MIC and MBC of onion extracts. A micro-broth dilution assay was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the tested onion extracts. The wells on the first and sixth column of a 96-well sterile microplate (BD Falcon; Fisher Scientific, Waltham, Massachusetts) were loaded with 200 µl of the extract to be tested. Two-fold dilutions of the extract solution were prepared by transferring 100 µl of the extract into adjacent wells containing 100 µl PBS from column 1 to column 5, and from column 6 to column 10. One hundred microliters of the prepared cocktail were loaded into reaction wells in columns 1-5 (final volume per well 200 µl), whereas wells in columns 6-10 remained not inoculated, to be used for establishing the baseline for the optical density (OD) reading at 630 nm. Final dilutions of the extracts should be 1:2,1:4,1:8,1:16,1:32, in wells 1-5 and 6-10, respectively. When the curve of OD for each dilution showed a flat line, the actual inhibition was determined by subtracting the minimum OD from the maximum OD obtained during the incubation period. This difference was named ∆OD. If the ∆OD was <0.05, then the dilution was considered to be inhibitory. The contents of wells with the dilutions showing inhibition were harvested and used to make dilutions and spread plating on TSA, incubating at 35°C for 24 h. After colony count, the bactericidal effect was defined as a count after incubation that was ≥3.0 log cycles lower than the initial concentration added to the well. The highest dilution showing bactericidal effect was the MBC.

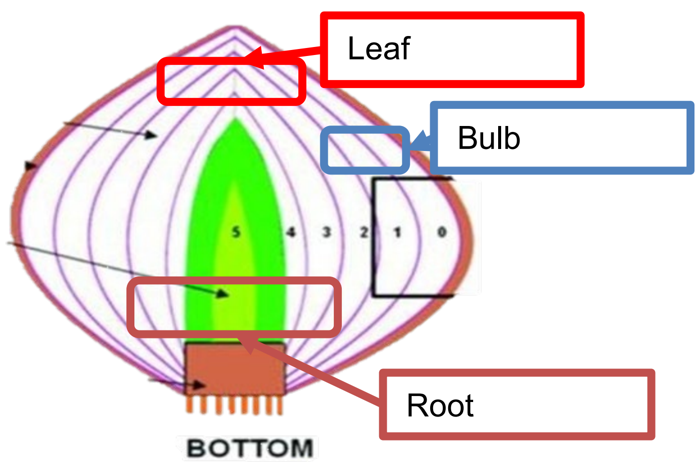
Survival of *Salmonella* in onion crude juices and onion bulbs. The *Salmonella* cocktail described above was used for the crude juice trials. For further challenge studies on onion bulbs, strains of *Salmonella enterica* subsp. *enterica* ser. Newport were isolated from clinical cases affected by the onion-linked outbreak in 2020 (CDC, 2020). All outbreak-related *Salmonella* Newport strains CC1070, CC1071, CC1072, and CC1073, were obtained from David Mann (University of Georgia - Center of Food Safety). Upon receipt, these isolates were individually streaked onto XLD to test for purity, and then individual colonies were transferred into 10 mL TSB and incubated for 24 h at 35°C. Preparation of frozen stocks with glycerol were done and stored in a freezer at -80°C for future use.

Onion sample preparation. Red, white, and yellow onion juices were prepared immediately following the preparation of the aforementioned cocktail to prevent oxidation. Onions were peeled and quartered using a flame-sanitized knife (Victorinox 10 in. Chef's Knife) and alcohol-sanitized cutting board (Faberware 11-in by 14 in White Poly) before being passed into a household juicer (Big Mouth Juice Extractor, Hamilton Beach, Glen Allen, Virginia). Roughly 500 ml of onion juice was collected from four large onion bulbs. The juicer was disassembled between onion varieties, and the machine was washed and then sanitized by submerging in a 10% bleach solution (Pure Bright Lancaster, Pennsylvania) for 10 minutes to prevent the carry-over of onion juices. Nine ml of onion juice was aliquoted into 14 sterile 15-ml centrifuge tubes. One milliliter of this inoculum was then added to each tube containing 9 ml of onion juice. Concurrently, 1 ml of inoculum was added to 9 ml of sterile TSB as a growth control and 9 ml of sterile PBS as a non-nutritive control. After inoculation, tubes were stored at 37°C in an incubator. At the beginning of storage (T0) and at 1-h intervals over 12 h, one tube of onion juice corresponding to each variety and the controls were randomly selected for plate count. A resuscitation method was followed to allow resuscitation of potentially injured cells, preventing potential count underestimation (Kang and Fung, 2000). Serial dilutions were prepared in buffered peptone water and spread-plated onto TSA. Plates were incubated at 37°C for 3 hours to allow injury repair, then overlayed by pouring molten xylose lysine decarboxylase (XLD) over the TSA surface. Plates were returned to the incubator to complete 24 h before counting colonies typical of *Salmonella*. This experiment was repeated in 3 independent trials. To determine the potential effect of pH on *Salmonella* survival, the pH of the juices was measured and recorded. Bagged red, white, and yellow onions were obtained from a local vendor a day before the experiment for challenge study and then stored in alcohol-sanitized plastic boxes at room temperature before the experiment.

Inoculum preparation and onion inoculation. A cocktail of the 4 *S.* Newport strains listed above was prepared. Each strain was placed in a 50 mL conical tube, centrifuged at 300 × g at 4°C for 15 min, and resuspended three times in PBS. All suspensions were then mixed, and the resulting cocktail was diluted to achieve a target final concentration of 5.0 log CFU/ml. This suspension was used to inoculate onions, and the actual bacterial concentration in the inoculum was confirmed by plate counts. On the day of the experiment, 90 bulbs of each onion variety were individually marked with food-grade ink by drawing 3 (three) 4 x 4 cm (16-cm^2) areas over the bulb surface. These marked surfaces (outer scale) were each spot inoculated with 100 µl of the inoculum. Additionally, another 100 µl of inoculum was injected into the bulb by puncturing with a loaded sterile tuberculin syringe (Becton Dickinson), inserting the full length of the syringe and releasing the inoculum. It was determined previously to achieve inoculation at the 3^{rd} scale inside the bulb. The inoculated bulbs were placed in storage boxes and left undisturbed at room temperature. Immediately after inoculation (zero time), and after 3, 6, 12, and 18 days, 6 bulbs from each onion variety were separated for sampling and testing.

Sample collection and *Salmonella* enumeration. To sample, the 3 outer scales were excised and composited in a single stomacher bag with 20 mL bag of buffered peptone water (BPW). To sample the inner scale, the external parts of the onion bulb were cut to expose the 3^{rd} scale under each of the 3 areas previously marked, which then was harvested by cutting approximately 16-cm^2. The 3 inner scale samples for each bulb were composited as described for the outer scales. Samples were homogenized in a stomacher at 230 RPM for 2 min, then decimal dilutions were prepared in buffered peptone water (BPW, Difco™, BD Life Sciences, Detroit, MI) and plated onto TSA plates, incubated at 37°C for 2-3 h and then overlaid with XLD agar to continue incubation over 18-24 h. Colonies typical of *Salmonella* were enumerated, and random colonies were picked to confirm their identity as *Salmonella*. Since no *Salmonella* was found on control onions, it was assumed that the counted salmonellae were the same as those inoculated.

Fluorescent *Salmonella* enumerations and microscopic examination. For this study, a strain of fluorescent *Salmonella enterica* subsp. *enterica* serotype Typhimurium (ATCC 14028GFP) was used to inoculate the onions. The selected strain expresses green fluorescence when grown in a medium with 100 ug/ml of Ampicillin for differentiation purposes. Forty-milliliter tubes of TSB with Ampicillin containing the reactivated microorganism were centrifuged at 3500 × g for 15 min at 4°C and then resuspended in phosphate buffer saline (PBS) solution three times. To enumerate the inoculum concentration, dilutions in 0.1% (wt/vol.) sterile peptone water (PW; Difco) were prepared and spread plated on TSA with Ampicillin to determine the inoculum concentration. The plates were incubated at 35°C for 18-24 h before colony counting. Potted onions of three different varieties (yellow, white, and red) were inoculated with fluorescent *Salmonella* from a suspension containing 9.4 log CFU/ml, at three different sites (Root, Bulb, Leaves), as described below. The onions were subjected to mild physical damage to promote internalization via injured tissue. Onion bulbs were lifted from the soil damaging the roots to inoculate the root. Bulbs were placed again in the pot and the surrounding soil was inoculated with 2 ml of *S.* Typhimurium ATCC 14028GFP. For microscopy, bulb sections close to the root site were dissected. The bulbs were inoculated by cutting with a scalpel blade at four different random places to mimic a bulb lesion; each bulb was inoculated with 2 ml of *S.* Typhimurium ATCC 14028GFP. Four microscopy bulb sections close to the lesion were dissected. To inoculate leaves, the leaves of each onion bulb were cut approximately 2-3 inches from the bulb, and 2 ml of *S.* Typhimurium ATCC 14028GFP was added. Four microscopy bulb sections close to the leaf site were dissected. The inoculation sites are depicted in the diagram below:



Tracking of *Salmonella* in onion-producing environments. To study the potential presence of *Salmonella* in onion-producing environments, sample plans were developed for preharvest and postharvest operations. In preharvest, two commercial and one experimental onion-growing fields were selected for sample collection in the Winter Garden area of Texas. Water samples were only collected from one commercial field since the second field's operator did not agree to water sampling. A total of 26 samples of irrigation water were collected from the well at one field, and 75 soil samples were collected from two commercial onion fields and one experimental field. The sampling was conducted at two commercial and one experimental packing facilities for postharvest operations. Onions grown and packed at the experimental facilities also entered commercial channels, therefore the experimental facilities were also treated as commercial. These packing plants were randomly named Plant A, B and C. A total of

154 samples were collected from various surfaces at Plants A, B and C during the 2023 onion season. Samplings were conducted in two separate sampling days for both pre and postharvest.

Preharvest sample collection. Before entering the onion fields, disposable plastic boot covers and non-powder latex gloves (Nasco, Fort Atkinson, WI) were sanitized with 70% ethanol and then worn to follow field policies on sanitation. To collect water samples, 50 L of well water (all fields used wells as water sources) was filtered through a sterile modified Moore swab (MMS) in a PVC cartridge. Previously, each MMS was made by cutting an 80 x 22 cm piece of grade #90 cheesecloth. The cheesecloth was rolled to allow a tight fit into an MMS cartridge made with 12 cm long, 4.4 cm diameter PVC pipe. The pipe was connected to a 2.54 cm male-to-male coupler on both ends, which is connected on one side to a 2.54 cm PVC connector. The cartridge was connected by silicone tubing measuring 0.25 x 0.44 cm on one end, whereas the other end had a couple to connect to the water valve of the well. The MMSs in their respective cartridge were wrapped in aluminum foil and autoclaved to sterilize. Previous trials allowed to determine the flow rate of water through, but this rate was again determined in the field due to a water pressure higher than expected. To collect the water sample, a hose was connected to one end of the sterile MMS cartridge, and the other to the water valve of the well. The water was then allowed to flow until filtering a volume of 50 L. The MMSs were transported to the Texas A&M AgriLife Research Center in Uvalde and then extracted from their cartridge using sterile forceps, placed each in a sterile Whirl-Pak bag (Nasco) containing 31 mL of BPW. Preliminary results showed that MMS retained 6 mL of water, hence, buffered peptone water concentration was adjusted accordingly to prevent dilution of the pre-enrichment broth. These bags were packed in refrigerated containers. Soil samples were collected using sterile scoops (Fisher Scientific, Hampton, NH), scooping up to 20 cm deep in the soil below the surface and some on the surface. From the size of the scoop, a sample of at least 1 kg of soil was expected. The soil was transferred to sterile Whirl-Pak bags, placed in refrigerated containers. The refrigerated containers with the water and soil samples were transported to the Food Microbiology laboratory in College Station, TX, for testing within 24 h after collection.

Postharvest sample collection. Non-food contact surface samples were collected from three packing plants. To collect these surface samples, sterile sponges (Sponge-Sticks, 3M™ Center, St. Paul, MN) were used to swab a 625-cm^2 area of walls, floors, fans, steps of stairs, and external sides of the onion graders at each packing plant. One of the plants also had a cooling room, from which three samples were collected from the wall, and one sample of an electric box was in one of the plants. No food-contact surfaces were collected from these plants. The dry sponges were hydrated with 26 mL BPW, removing excess liquid from the swabs by gently squeezing. Each of the identified surfaces, i.e., floors, walls, fans, external side of graders, and stair steps in each plant, were sampled by vigorously rubbing with the moist sponge 10 times diagonally, vertically, and horizontally in each of the directions. After the initial swabbing on one side of the swab, the same was done on the opposite side of the swab, and finally completed using the front edge of an individual swab. The handle of the swab is gently detached and removed after the swab is returned to the sampling bag. All bags containing the surface samples were placed in a refrigerated container to be transported to the food microbiology laboratory in College Station for testing within 24 h after collection.

Sample processing. All samples were placed in ice chests (Igloo™, Katy, TX) containing ice packs and transported back to the Food Microbiology Laboratory at the Department of Animal Science, Texas A&M University (College Station, TX) on the same day and kept in the cold room at 4-8°C overnight before experiments. Soil samples were thoroughly hand-mixed inside their bag, vigorously rubbing and shaking the ~1,000-g sample for 1 min. Then, 25 g of each sample was combined with 26 mL BPW in a sterile filter Whirl-Pak bag, hand massaged for 2 min, and squeezed to obtain a clear solution. One milliliter of the contents was then used to prepare decimal dilutions to enumerate *Enterobacteriaceae*; the remaining 25 mL were used for *Salmonella* detection and quantification. For onion-packing plant surfaces, the sponges were hand massaged inside their bag for 2 min to homogenize with the 26 ml of BPW, and then squeezed to collect 1 ml, which was used for serial dilutions and enumeration of *Enterobacteriaceae*. The remaining 25 mL was used for *Salmonella* detection and quantification.

***Salmonella* assay.** All samples were tested for presence and numbers of *Salmonella*. Both the qualitative and quantitative *Salmonella* assay was conducted in a BAX® system, with SalQuant® add-on for the *Salmonella* counts. For water and onion-packing plant surfaces, the homogenates containing the sample and 25 ml of BPW were added to 225 mL pre-warmed BPW. For soil samples, 25 g of each mixed soil sample was added to 225 mL pre-warmed BPW and mixed in a stomacher. Of this, 30 mL of each sample were transferred to sterile Whirl-Pak bags and mixed with 30 mL BAX MP media + 1 mL/L Quant solution (Hygiena) pre-warmed at 42 ± 1 °C. The remaining sample was incubated at 42 ± 1 °C for 18-24 h for later confirmation of *Salmonella* if testing positive. The sample with the MAX-MP media was incubated at 42 ± 1 °C for 15-24 h, then homogenized by hand for 2 min. Five microliters of this enriched sample was added to 200 mL of lysis reagent containing protease enzyme in the corresponding cluster tube, which is prepared by labeling and arranging in rack according to the rack file that lists sample names in the BAX®-System-SalQuant® Q7 application. Bacterial DNA lysis was performed by heating the tubes at 37 and 95°C in an Automated Thermal Block (Hygiena, Camarillo, CA) for 20 min and 10 min, respectively, then cooling for 5 min at 2-8°C in a cooling block. After arranging the PCR tubes according to the rack file, 30 mL of lysate was transferred into the PCR tubes and sealed with flat optical caps. This was repeated for all remaining strips of the PCR tablets for hydration. The BAX Q7 System achieved the detection (presence/ absence) of *Salmonella*. The possible outcomes of the results were negative (-), positive (+), or indeterminate. Samples testing positive (absence/presence) for *Salmonella* in the first 6 h of sample incubation (detection) were subjected to the same BAX procedure as for detection, the only difference was incubation for enrichment will be done 18-24 h at 42 ± 1 °C. The SalQuant assay yielded a Cycle Threshold (CT) value, which was used in the SalQuant Calculator (Hygiena, Camarillo, CA) to determine the *Salmonella* concentration.

Confirmation of *Salmonella*. If present (positive) after BAX®-System-SalQuant® confirmation, the enriched sample was plated on XLD agar and incubated. From these plates, one colony representing a typical *Salmonella* morphology per sample was picked and transferred to lysine iron (LIA) and triple sugar iron (TSI) agar slants (Hardy Diagnostics, Santa Maria, CA), inoculating by butt stabbing and slant streaking using a sterile needle. The slants were incubated at 37 ± 2 °C for 18-24 h. Positive slants for *Salmonella* were confirmed by agglutination with polyoxetine (Difco) following MLG 4.14 guidelines (US Department of Agriculture – Food Safety Inspection Service, 2023).

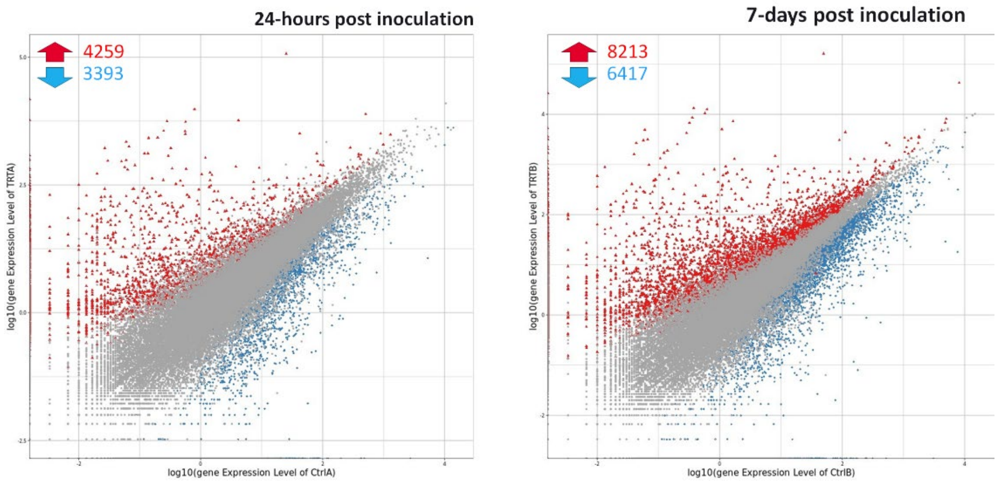
***Enterobacteriaceae* counts.** As indicated above, all samples were tested for *Enterobacteriaceae* (EB) counts by plate counting using Enterobacteriaceae Petrifilm® (3M™ Center, St. Paul, MN). For EB enumeration, 1 mL of each sample was serially diluted in buffered peptone water (BPW), and mixed on a vortex mixer (VF2, IKA, Hamburg, Germany). For each selected dilution, 1 ml was added onto the EB Petrifilm®, using the Petrifilm spreader to ensure coverage of the countable area, then incubating at 37 ± 1 °C for 24 ± 2 h.

Onion bulb surface characterization.
Atomic Force Microscopy (AFM): The NanoTechnology AFM equipment (Material and Characterization Facility, Texas A&M University) was used to acquire the AFM images of the onion's outer and inner layers (inner layer viewing somehow proved futile due to their wetness) in contact mode at room temperature in a UHV environment ($4\times10{-}9$ Pa). To prevent scratching the crystal surface, we maintained a minimal contact force (up to 2-4 nN) for all photos taken in the constant force mode. To obtain the topographic images, silicon cantilever Nanosensors Pointprobe (force constant ~0.2 N/m) with a polygon-based pyramid-shaped tip and tip radius smaller than 7 nm was utilized. Images allowed for obtaining the roughness values (Rq mean).

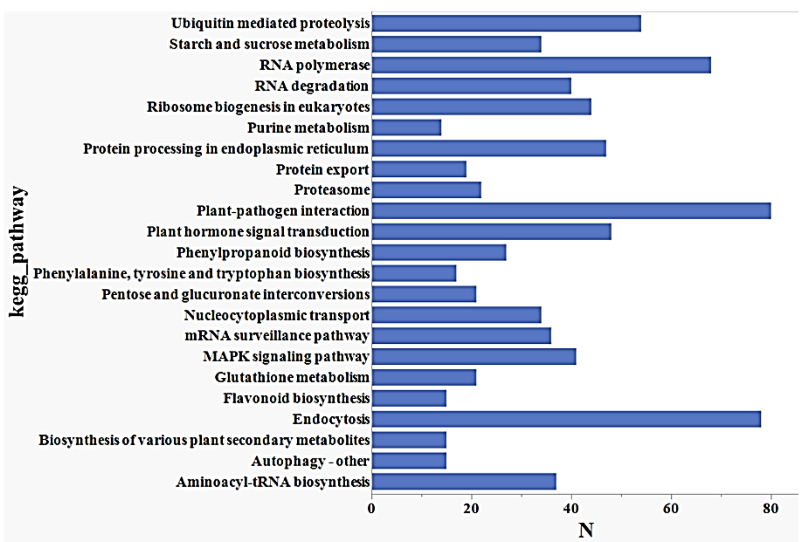
Scanning Electron Microscopy (SEM): The JEOL JSM-7500F, an ultra-high resolution field emission scanning electron microscope was used. Using double-sided adhesive tape, a section of the outer papery onion sample of each cultivar was mounted onto small sample holders designed to fit onto the stage of the microscope; they were fixed and left to dehydrate and then the next day they were sputter coated before viewing. All experiments were conducted with an accelerating voltage of 20 kV, a working distance of 8.5 mm, and a beam diameter of 20 nm at different magnifications. Protocols to mitigate beam damage were adhered to.

Results

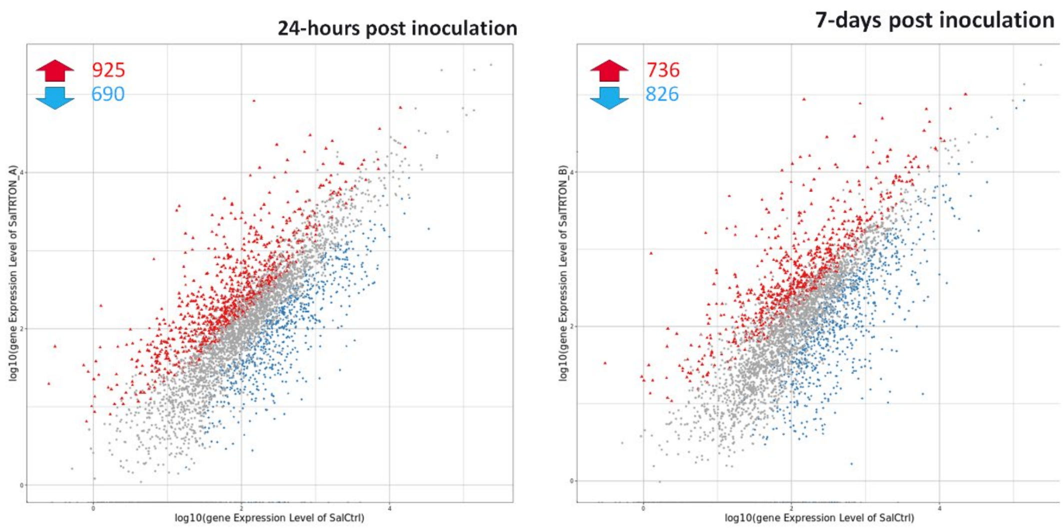
Transcriptomic changes in the onion bulb and *Salmonella* during their interactions:
Transcriptomic analysis of onion bulb tissue (variety Don Victor) in response to *Salmonella* was performed 24 hours and 7 days post-inoculation to identify differentially expressed genes (DEGs) in onion. Within 24 hours of inoculation, 4259 genes were upregulated while 3393 genes were downregulated. After 7 days, 8213 and 6417 genes were up and down-regulated, respectively, as shown below. (FDR<0.05 and Abs (log 2(Y/X) >0)

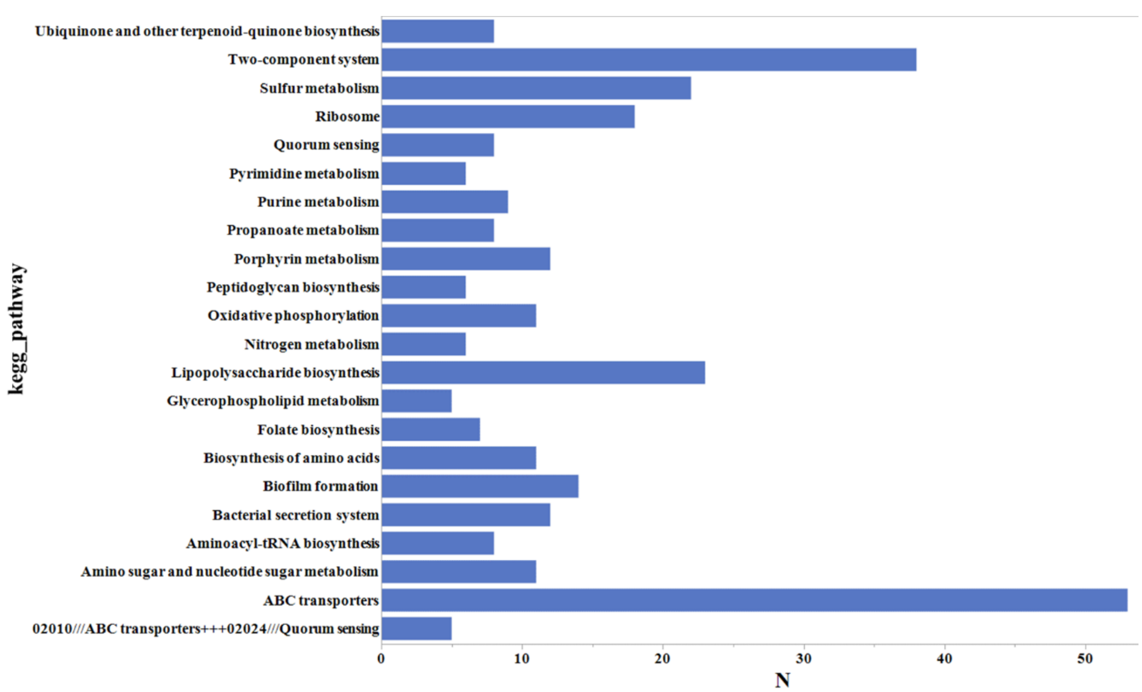


The number of genes that were differentially expressed was dominated by the genes involved in plant immunity responses. The KEGG pathway analysis of these genes' associated functions (see below) primarily represented the genes related to plant-pathogen interaction and endocytosis-associated processes like nutrient uptake, signaling transduction, or plant-microbe interactions.

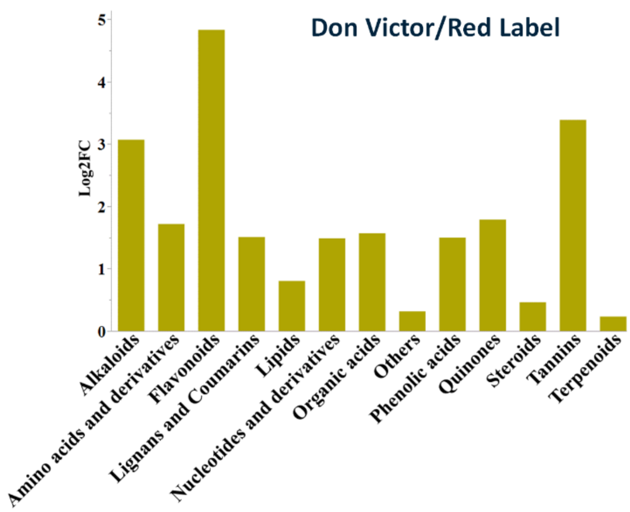


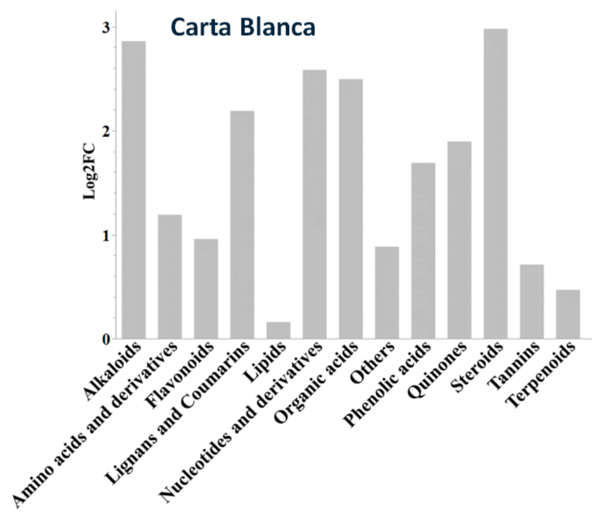
Similarly, we used dual sequencing to look at the transcriptomic changes in the inoculated *Salmonella* in the onion bulb after 24 hours and 7 days to understand how *Salmonella* transcriptome responds during interaction with the onion bulb, especially to its induced metabolites and immunity-responsive genes. 925 and 736 genes were upregulated within 24 hours and 7 days after inoculation, while the number of downregulated genes in the same timeframe was 690 and 826, respectively (see below). (FDR<0.05 and Abs (log 2(Y/X) >0). The KEGG pathway analysis of these genes' associated functions suggested differential expression of the genes related to the regulators of systemic infection, pathogenicity island or components of the Type III secretion system, implying the active involvement of hallmark genes required by *Salmonella* to establish colonization.



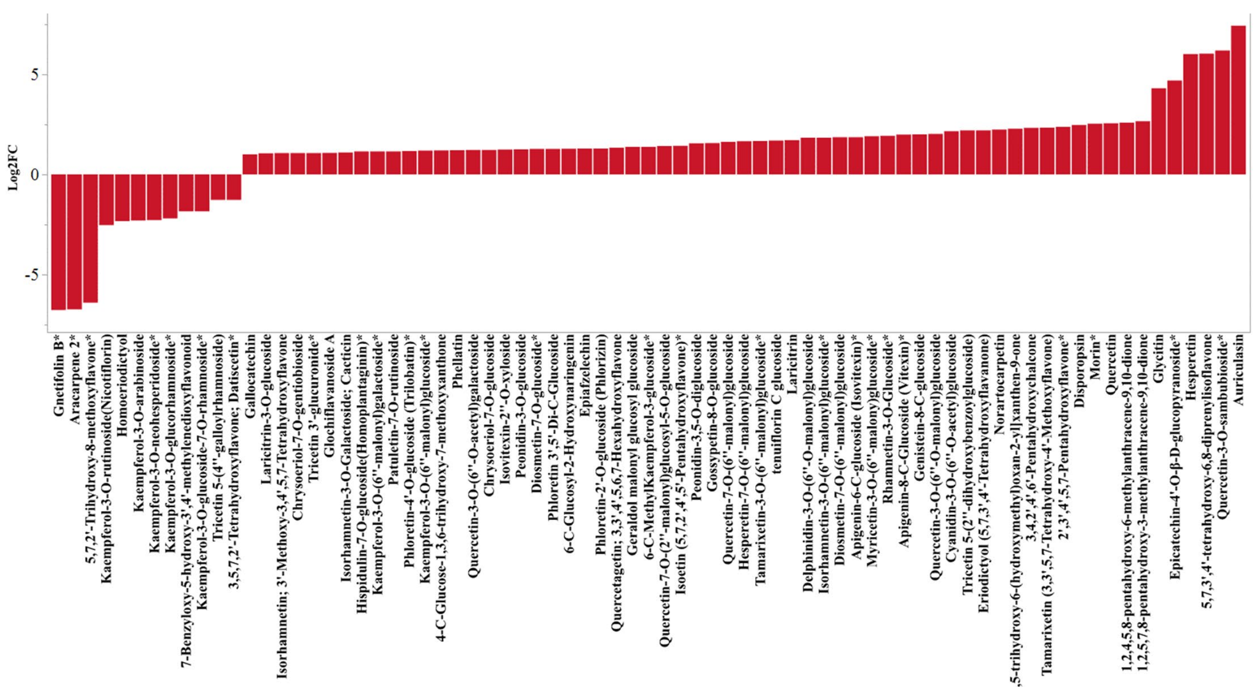


Metabolic changes in the onion bulbs induced in response to *Salmonella* inoculations: Differentially expressed metabolites (DIMs) were detected using widely targeted metabolomics by UPLC-MS/MS analysis. Tissues collected within 24 hours and 7 days after inoculation from three varieties representing three colors (Red Label – red, Don Victor – yellow, and Carta Blanca – white) were used for the analysis. Although the flavonoids dominated in the case of red/yellow varieties, several-fold changes in different groups of metabolite families were recorded in response to *Salmonella* inoculation (see below). (Additional data of each metabolite will be included in a publication under preparation.)





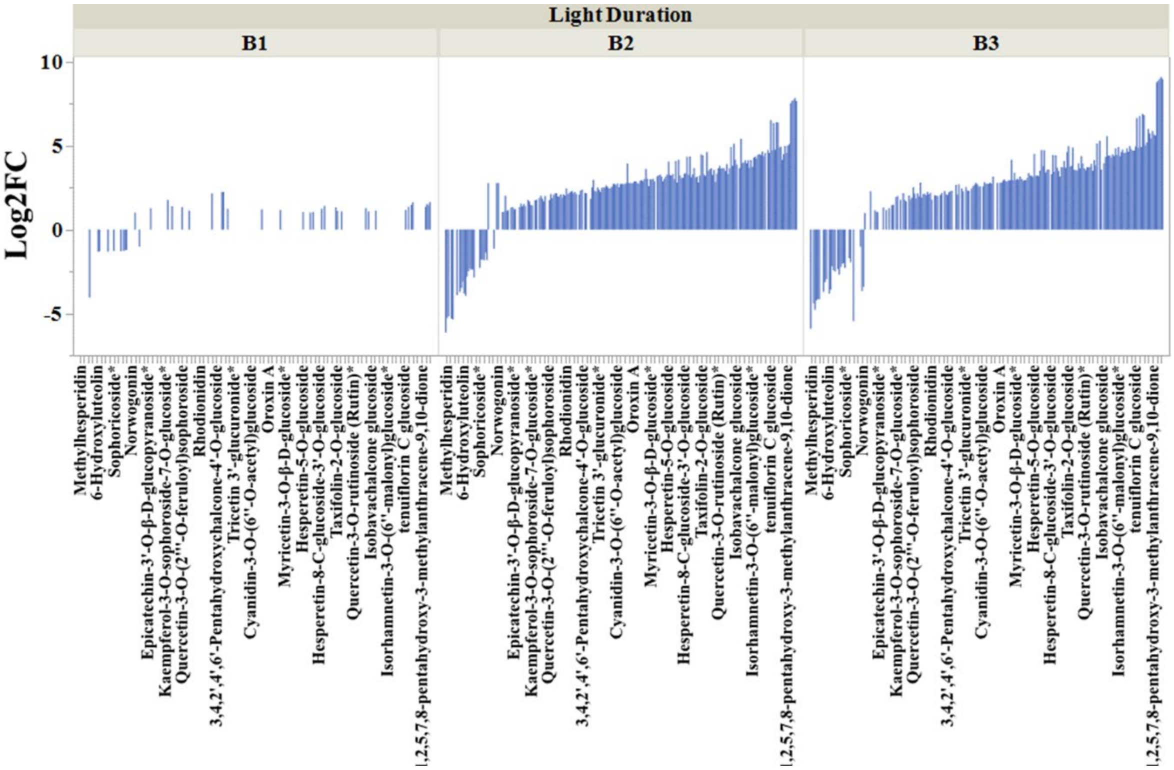
Among the flavonoids, quercetin and its derivatives showed the most potent increase up 10-fold induction within 24 hours of inoculation for yellow and red varieties. A representative graph of selected quercetin and its derivatives in the Red Label variety is shown below.



Effect of onion components on the survival of *Salmonella*: The extracts from Carta Blanca (white) and Dulciana (yellow) across all concentrations did not inhibit but permitted the growth of *Salmonella*. As such, no MIC or MBC could be established for the extracts of these two varieties. Nevertheless, **Table 1** shows the optical densities for extracts of yellow, white, and red onions grown under different conditions of water and soil nitrogen concentrations. No differences were observed in the OD readings for white and red onion extracts, but for yellow onion extracts, the OD was significantly higher for low water-low nitrogen conditions. Paradoxically, the high water and high nitrogen conditions also resulted in the more remarkable growth of *Salmonella*. Therefore, it cannot be concluded that the microorganism was able to grow at a higher rate under water-stress conditions.

Regarding Red Label (red) onion extracts, *Salmonella* growth was inhibited across multiple dilutions of the extracts. The ∆OD values for each observed inhibitory dilution are in **Table 2**. The MIC and MBC of each extract are recorded in **Table 3**, and all data for Red Label onions is shown in **Table 4**. There seem to be no apparent differences in the MIC or MBC for red onions grown under various nitrogen content and water conditions, possibly indicating that the exposure to water or nitrogen stress does not result in a more significant accumulation of AOC, which would be expected as a defense mechanism of the plant when under stress. However, when comparing the OD for all extracts, regardless of their inhibitory effect, the effects of water and nitrogen were independent, as seen in **Table 5**. Although yellow and white onion extracts did not inhibit *Salmonella* even at the highest concentration, their growth was still affected by the concentration, showing that the higher the concentration, the slower the growth (**Figure 1**). Therefore, the effect of nitrogen and water content in the soil was compared for all varieties. Low nitrogen was determined to be statistically significant across all tested onion extracts. The interaction of water and nitrogen in the Red Label extracts only, as they were the only ones to cause inhibition, determined the effect of the soil conditions to be dependent on one another. These comparisons were made using the MIC values, as the Red Label extracts successfully inhibited the *Salmonella* growth. These results are shown in **Table 6**. The lack of inhibition was observed regardless of the onion layer used for the extracts.

Effect of growing conditions and genotype on the inhibition capacity of onion extracts: The impact of growing onions under the light of various wavelengths showed that the light color may influence the potential development of inhibitory compounds. **Figure 2** shows extracts from red and white onions grown under blue light, producing inhibition of *Salmonella*. In contrast, extracts from onions grown under white light did not show any inhibition across all varieties. In contrast, red light resulted in extracts of mild to no inhibition, depending on the onion variety. It was evident that lighting during the greenhouse growing of onions affects the plant's metabolism, likely leading to more efficient production of potentially antimicrobial compounds. The metabolite analysis, particularly for flavonoids, further confirmed that the red and far-red spectrum enhanced flavonoid accumulation over time (see image below; the data is shown for the red onion variety, Sofire, exposed to the light treatments after harvest; B1>B2>B3 10-day interval).



Regarding the effect of the onion genotype on the inhibitory activity of the extracts, or 12 genotypes tested, only one (1104) showed OD significantly lower than all other extracts. Genotype 544X showed significantly lower OD than the extracts of the remaining ten genotypes; however, this still showed minimal growth (**Table 7**). **Figure 3** shows the effect of layer type in genotype 1104 on microbial growth of *Salmonella*. The outer layer inhibited growth more than the inner layer, as both differed significantly from the mean. However, this significant difference seemed to be numerically small, leading to uncertainties about whether the layer would have a biological effect. Further research described below confirmed this observation.

Survival of *Salmonella* in onion juice: Juices made from all onion varieties showed an inhibitory effect and produced death curves with various death rates. In all cases, initial *Salmonella* concentrations of 6-7 log/ml were reduced to close to or below the detection limit of 0 log CFU/ml after 12 h storage at room temperature (20°C). **Figure 4** shows the death curves and the lag time, expressed as the time to a reduction phase. These times were 1.2, 5.4, and 2.1 h for yellow, white, and red onion juices, respectively, indicating a likely difference in the toxicity levels of juices extracted from different onion varieties. **Table 8** shows the calculated D-values for *Salmonella* in the juices. These values are approximate based on Baranyi's model and only represent a single segment of the sigmoid curve. However, they show that even though all juices were inhibitory, the reduction rate varies according to the variety, indicating differences in the toxicity of the antimicrobial compounds or attenuating effect of other onion components that may interfere with the antimicrobial effect of the active compounds. The pH for red, white, and yellow onion juices was 5.43, 5.46, and 5.34, respectively. This indicates that the reduction observed in *Salmonella* may not be due to a pH effect.

Survival of *Salmonella* in and on onion bulbs: *Salmonella* Newport was able to grow in the inner layers of all onions in the challenge studies, reaching a stationary phase in 3 days on white and yellow onions, whereas on red onions, *S.* Newport continued slow growth to reach ~8 log CFU/100 cm^2 of inner scale surface. In contrast, on the outer scales of all onion varieties, *S.* Newport was reduced in all onions by 2-3 log cycles within three days of storage (**Figure 5**). This confirms previous findings where extracts made from the inner scale were not inhibitory, whereas the extracts made from the outer scales inhibited the growth of *Salmonella* during microplate assays.

Allocation of *Salmonella* in onion structures: Fluorescent *S.* Typhimurium survived in onions inoculated internally for 7 days (168 h) during the latest stages of onion cultivation in pots. Multifactorial ANOVA indicated no significant change in counts of fluorescent *S.* Typhimurium inoculated internally. In contrast, this microorganism decreased significantly over 168 hours in externally contaminated onions, particularly in red and yellow onions. In contrast, a significant reduction was seen in white onion after 24 h, but there was no further reduction after 168 h (data shown in **Figure 6**). Again, the survival and even growth of *Salmonella* in internal structures shows the food safety significance *Salmonella* can internalize the onions. Fluorescence microscopy analysis showed that *Salmonella* can spread in internal tissues. **Figures 7 and 8** show fluorescent cells being allocated around the protoplasm of onion cells and cells being internalized in the stomata after tissue damage.

Tracking of *Salmonella* in onion-producing environments (Prevalence of *Salmonella*): *Salmonella* was detected in 3 (13%) of 23 samples of well water collected from one onion field, 3 (4%) of 75 samples of soil collected from three fields, and 16 (10%) of 151 surface samples collected from three onion packing plants (**Table 9**). The samples that tested positive were subjected to quantitative analysis. Of the water and soil samples collected in the onion field that tested positive for *Salmonella*, the mean *Salmonella* counts were 0.1 log CFU/50 L in water and 1.2 log CFU/g in soil. The low count of *Salmonella* in water, equivalent to 25 CFU/m3, highlights the importance of an adequate sample size to be collected. The quantitative data are shown in **Table 10**. Most of the *Salmonella*-positive samples were from walls (20%), floors (13%), and fans (13%) (**Figure 9**). The number of *Salmonella*-positive samples from plants A, B, and C was 4 (7%), 12 (21%), and 0 (0%), respectively. The percentage of positive samples for plant B was significantly higher than those for plants A and C ($P < 0.01$). The counts of *Salmonella* on the 16 positive surface samples are shown in **Table 11**. The means ranged between 1.9 and 3.2 log *Salmonella* CFU/cm^2 on 625-cm^2 surfaces. However, there was a large variability in counts for all sample types. For example, on walls, the counts ranged from as low as -3.4 log CFU/cm^2 (equivalent to 4 CFU 6.1 per 100 m^2) to as high as 6.1 log CFU/cm^2. These findings are an indication of the need for better sanitation strategies to be applied in these onion-packing plants. Interestingly, surfaces adjacent to food-contact surfaces, such as the outside of grade machines, tested consistently negative, which may indicate that sanitation may be applied to processing areas with obvious need for sanitation, while other surfaces away from the food contact, such as fans and walls may be neglected.

***Enterobacteriaceae* (EB) counts:** In onion fields, one field producer allowed us to collect irrigation water from the source. In this case, the EB counts ranged between 0.8 and 2.4 log CFU/ml, with a mean of 1.7 log CFU/ml. In soil, these counts ranged between 1.0 and 5.8 log CFU/g, with a mean of 3.9 log CFU/g. The presence of EB in the field is most likely not relevant since these organisms are natural elements of the field microbiota, and therefore, their presence is expected. In contrast, EB's presence on packing plant surfaces may be used as an indicator of adherence to sanitation procedures. Overall counts of EB obtained from surface samples were 2.5, 1.7 and 2.7 log CFU/cm2 at packing plants A, B, and C, respectively. Plant B produced EB counts significantly lower than plants A and C (**Figure 10**). However, absence of a baseline for these counts on packing surfaces makes it difficult to conclude whether the level of sanitation was acceptable based on EB counts. These results then will only compare levels found on different types of surfaces without referring to levels of hygiene. The counts by type of surface and packing plant are shown in **Table 12**. No differences were found between packing plants when breaking down to surface type. In all cases, floors showed the highest counts.

Onion bulb surface characterization: Onions were tested for roughness and topography using atomic force microscopy (AFM) and scanning electron microscopy (SEM), respectively, to see how these factors affect the internalization of *Salmonella* in three varieties of onions. For SEM, only the outer papery layer was viewed because wet materials cannot be used in such a technique. For AFM we viewed both the outer layer(s) and inner wet layers (layer just adjacent to the first layer; the rest were also too wet for AFM). Results (**Table 13**; **Figures 11, 12, and13**) show that the layers of the white variety were rougher, followed by yellow and red in that order. These results suggest that color, which is a result of anthocyanins, could have a direct correlation with how soft or less porous the onion variety surface is and that the less color, the higher the likelihood that it will harbor *Salmonella* in its structures and vice versa.

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Outcomes and Accomplishments

The dual transcriptomic analysis confirmed the differential expression of onion genes associated with plant innate immunity in response to *Salmonella*, while the induction of genes related to *Salmonella*'s Type III secretion system suggests its ability to counteract the onion bulbs induced defense responses and activating mechanisms to colonize onion bulb scales. The metabolomic analysis confirmed the activation of several defense-related metabolites, particularly flavonoids like quercetin and its derivatives in onion bulbs, known for their antimicrobial properties within 24 hours after inoculation with *Salmonella* in the case of red and yellow onions. Significant differences in the inhibitory activity against *Salmonella* among selected onion varieties suggest the possibility of breeding onion varieties that could be more tolerant to foodborne diseases.

The results of this study permitted an understanding of non-evident issues related to onion safety. Although the lower antimicrobial effect in the internal tissues of onion bulbs had been reported, this study allowed a more in-depth evaluation of this effect. We have contributed to a better understanding of the implications of internalization of bacterial pathogens in onion bulbs. We proved that onions can inhibit *Salmonella* growth and even reduce this pathogen, but multiple factors may hinder the expression of this antimicrobial effect. The use of alcoholic extracts may require further review to ensure an accurate view of the activity of quercetin and other phenolics as mechanisms to enhance the immunity in onion bulbs and reduce *Salmonella* effectively.

Another accomplishment was quantifying the magnitude of growth and dissemination of *Salmonella* when internalizing onion bulbs. Although preventing produce damage is common knowledge, the effect of pathogen internalization resulting from tissue damage was confirmed and quantified. The importance of light-induced changes in the flavonoid content during the storage period for onion bulbs was highlighted, which should help design further research.

With regard to field studies tracking the presence and numbers of *Salmonella* in onion production environments, this was the first quantitative study of this pathogen in the agricultural environment, which may be extrapolated to other commodities. We generated data that should assist in assessing the risk of onion contamination from water and soil, and by using a large sample approach (50 L of water, for example), we were able to detect *Salmonella* in minimal concentrations, which can be evaluated to determine whether this would pose a risk for onion contamination or not. Testing agricultural water for pathogens using this large sample approach is another item that we intend to continue exploring for onion and other produce commodities. Lastly, we were also able to quantify the prevalence of *Salmonella* on non-food contact surfaces at onion packing facilities and identified a possible need for developing adequate sanitation programs, including evaluation of procedures and training.

At this time, the one measurable outcome of this study is this final report, but we are working on more outcomes, such as peer-reviewed publications as well as meetings with the onion industry to assist in the form of developing sanitation SOP as well as possible training.

Summary of Findings and Recommendations

1. Transcriptomic responses induced in onion bulbs within 24 hours and after 7 days demonstrate the role of plant-induced immunity and, hence, the ability of plants to recognize and counteract the invasion of *Salmonella* like a plant pathogen. The genotypic differences in the inhibitory potential further justify the possibility of developing varieties tolerant to *Salmonella* contamination.
2. The metabolomic analysis of onion bulbs exposed to *Salmonella* resulted in differential induction of several metabolites representing different functional classes. Quercetin and its derivatives, known for their antibacterial properties, showed consistently several-fold induction in red and yellow varieties.
3. The entry routes of *Salmonella* through injured roots, leaves, and scales and their localization around stomata studied using GFP fluorescence imaging suggest *Salmonella*'s ability to move inside the bulb and its ability to survive within bulb tissue, which is critical to understanding and devising the detection strategies.
4. The onion variety affects the antimicrobial effect of active components such as flavonoids and polyphenols. However, all onions may show inhibitory or lethal effects against *Salmonella* depending on how the onion tissue is processed.
5. Red onions have a greater concentration of active compounds showing an inhibitory effect, reflected in the more significant inhibitory effect during extract testing. However, other forms of preparations, such as crude extracts, show a different image.
6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods used for evaluating the inhibitory effect of onion extracts may show a potentially false lack of impact on onions that are not red.
7. The antimicrobial components of the onion seem to be located at greater concentration on the external scales, and the inhibitory effect is almost non-existent in the internal tissues of the onion. This highlights the importance of protecting onion bulbs from physical damage. If *Salmonella* can internalize, it can grow and spread inside the onion bulb.
8. The isolation of *Salmonella* from non-food contact surfaces in packing plants evidences the need to implement adequate plant sanitation programs. Further training for onion packing personnel and developing sanitation SOPs is recommended. Adopting environmental monitoring programs to verify the effectiveness of cleaning and sanitizing is encouraged.

APPENDICES

Publications and Presentations

Manuscripts in preparation

- Duel Transcriptomic sequencing suggests the role of plant innate immunity during onion bulb-Salmonella interaction (Under preparation). Joshi and Castillo et al.

- Widely targeted metabolite profiling indicates the involvement of quercetin and other flavonoids in bulb onions in response to Salmonella (Under preparation). Joshi and Castillo et al.

Presentations
Joshi, V. Oral Presentation "New insights into the interactions between bulb onions and Salmonella" at the Department of Horticultural Sciences Summer Seminar series, College Station, November 2, 2023.

Budget Summary

This project was awarded a total of $408,522. The majority of funds were spent, and remaining funds will be used for travel to the CPS Research Symposium.

Tables 1–13 and Figures 1–13 (see below)

Table 1. Growth/survival of *Salmonella* in onion extracts as affected by the amount of irrigation water and level of nitrogen in the soil during growth[a].

Variety	Water level	Nitrogen level	LS Means OD[b]	
Dulciana (Yellow)	Low	Low	0.512	A[c]
		High	0.439	B
	High	Low	0.442	B
		High	0.506	A
Carta Blanca (White)	Low	Low	0.473	AB
		High	0.460	AB
	High	Low	0.460	AB
		High	0.479	AB
Red Label (Red)	Low	Low	0.004	C
		High	0.009	C
	High	Low	0.002	C
		High	0.003	C

[a] SEM = 0.01.
[b] OD, Optical density at 360 nm.
[c] Means followed by same letter are not significantly different ($p < 0.05$).

Table 2. Differences (∆) between maximum and minimum optical density (OD) for various dilutions of Red Label onion extracts and their corresponding soil conditions[a].

Extract[c]	Dilution titer	OD[b]		∆OD[d]
		Minimum	Maximum	
HW-HN	1:02	-0.004	-0.002	0.002
	1:04	-0.004	0.000	0.004
	1:08	-0.002	0.002	0.004
	1:16	-0.002	0.003	0.005
	1:32	-0.002	0.001	0.003
HW-LN	1:08	-0.004	0.003	0.007
	1:16	-0.001	0.001	0.002
	1:32	-0.002	0.052	0.054
LW-HN	1:02	0.000	0.005	0.005
	1:04	-0.003	0.004	0.007
	1:08	-0.002	0.003	0.005
	1:16	-0.003	0.085	0.088
	1:32	-0.001	0.000	0.001
LW-LN	1:04	-0.003	0.001	0.004
	1:08	-0.001	0.002	0.003
	1:16	-0.001	0.003	0.004
	1:32	-0.002	0.016	0.018

[a] HW- High Water, LW- Low Water, HN- High Nitrogen, LN- Low Nitrogen.
[b] For each dilution, the maximum and minimum OD obtained from hourly measurements during 24 h.
[c] H- optimal conditions, L- Induced stress by reduced water/ nitrogen.
[d] Maximum OD − minimum OD = ∆OD; threshold of inhibition- 0.05.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for extracts of red onions (var. Red Label) grown under various soil water and nitrogen contents[a].

Effect (Water by Nitrogen)[b]	Least Square Means	
	MIC (mg/g W/W)	MBC (mg/g W/W)
HW*HN	0.24 A[c]	0.57 A
HW*LN	0.18 A	0.26 A
LW*HN	0.32 A	0.94 A
LW*LN	0.26 A	1.06 A
SEM	0.086	0.230

[a] HW- High Water, LW- Low Water, HN- High Nitrogen, LN- Low Nitrogen.
[b] H- optimal conditions, L- Induced stress by reduced water/ nitrogen.
[c] Within columns, values followed by the same letters are not significantly different ($p<0.05$).

Table 4. Mean, standard deviation, standard error, and 95% confidence intervals of OD values from inhibitory concentrations of Red Label onion extracts of different soil conditions[a].

Extract[b]	Concentration	Number of Measurements	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
HW-HN	1.293	25	-0.002	0.0006	0.0001	-0.003	-0.002
	0.647	50	-0.001	0.001	0.0001	-0.001	-0.001
	0.323	75	0.0001	0.001	0.0002	-0.0002	0.0005
	0.1617	125	0.0002	0.002	0.0002	-0.0001	0.0006
	0.0808	50	-0.0009	0.001	0.0001	-0.001	-0.001
HW-LN	0.468	75	-0.0001	0.002	0.0002	-0.001	0.0004
	0.2340	100	-0.0001	0.0004	0.00004	-0.0001	0.00003
	0.1170	75	0.008	0.02	0.002	0.004	0.01
LW-HN	1.862	25	0.001	0.001	0.0002	0.001	0.002
	0.931	100	0.001	0.002	0.0002	0.0002	0.001
	0.466	125	0.001	0.001	9E-05	0.001	0.001
	0.2328	75	0.002	0.01	0.001	-0.0001	0.01
	0.1164	25	-0.0002	0.0004	8E-05	-0.0004	0
LW-LN	1.411	75	-0.0006	0.002	0.0002	-0.001	-0.0003
	0.706	150	0.0001	0.001	6E-05	0	0.0003
	0.3528	125	-0.0001	0.001	0.0001	-0.0003	0.0001
	0.1764	50	0.004	0.01	0.001	0.002	0.0

[a] HW- High Water, LW- Low Water, HN- High Nitrogen, LN- Low Nitrogen.
[b] H- optimal conditions, L- Induced stress by reduced water/ nitrogen.

Table 5. Soil conditions on the optical density of Red Label, Dulciana and Carta Blanca extracts[a].

Effect[b]		Least Sq Mean	Standard Error	Significance[c]
Water by Nitrogen	HW*HN	0.19	± 0.012	A
	HW*LN	0.16	± 0.011	A
	LW*HN	0.20	± 0.012	A
	LW*LN	0.16	± 0.013	A
Water	HW	0.17	± 0.008	A
	LW	0.18	± 0.008	A
Nitrogen	HN	0.19	± 0.009	A
	LN	0.16	± 0.008	B

[a] HW- High Water, LW- Low Water, HN- High Nitrogen, LN- Low Nitrogen.
[b] H- optimal conditions, L- Induced stress by reduced water/ nitrogen.
[c] Least sq means followed by the same letters are not significantly different ($p<0.05$).

Table 6. Soil conditions on the MIC of Red Label extracts[a].

Effect[b]		Least Sq Mean	Standard Error	Significance[c]
Water by Nitrogen	HW*HN	0.000	± 0.0003	C
	HW*LN	0.001	± 0.0003	B
	LW*HN	0.002	± 0.0003	A
	LW*LN	0.000	± 0.0003	BC

[a] HW- High Water, LW- Low Water, HN- High Nitrogen, LN- Low Nitrogen.
[b] H- optimal conditions, L- Induced stress by reduced water/ nitrogen.
[c] Least sq means followed by the same letters are not significantly different ($p < 0.05$).

Table 7. Effect of the onion genotype on the growth of *Salmonella* in onion extracts

Genotype	Means[a]
218Y	0.68 ± 0.38 A[b]
541X	0.68 ± 0.38 A
1196	0.64 ± 0.37 AB
Yellow H6	0.64 ± 0.39 ABC
545X	0.62 ± 0.40 ABC
25X	0.61 ± 0.38 ABC
542	0.60 ± 0.41 BCD
1197	0.59 ± 0.37 BCD
1102	0.57 ± 0.38 CD
231Y	0.53 ± 0.32 D
544X	0.45 ± 0.29 E
1104	0.33 ± 0.36 F
Ampicillin[c]	0.16 ± 0.29 G

[a] Means represents optic density measured at λ 630 nm.
[b] Means followed by same letter are not significantly different ($p < 0.05$).
[c] Ampicillin was used as positive control to ensure inhibition.

Table 8. Maximum reduction rates of *Salmonella* in raw onion juices during storage at 25°C

Parameter	Yellow	White	Red
Log T_0-Log T_7	4.8[a]	4.2	6.0
Slope[b]	-0.795	-2.188	-1.459
D[c]	1.258	0.457	0.686

[a] Log CFU/ml at zero time – log CFU/ml at 7 h storage.
[b] Slope within segment of the curve showing a straight descending line.
[c] D, time to 1 log reduction (in h).

Table 9. Incidence of *Salmonella* in onion growing and packing environments

			Positives	
Source of samples	Type of sample	No. tested	No. samples	%
Fields	Water	23	3	13A[a]
	Soil	75	3	4A
Packing plants	Non-contact surfaces	154	16	10A

[a] Percent values followed by same letter are not significantly different ($P > 0.05$).

Table 10. Counts of *Salmonella* in samples of water and soil collected from onion fields, which tested positive for qualitative analysis[a]

Type of sample[b]	Positive sample #	*Salmonella* CFU/volume or weight	*Salmonella* Log CFU/volume or weight[c]
Water	1	1.0	0.0
	2	0.8	-0.1
	3	2.1	0.3
	Means	1.3	0.1
Soil	1	5.2	0.7
	2	7.1	0.9
	3	110.0	2.0
	Means	41.0	1.2

[a] Both quantitative and qualitative tests were conducted using Bax SalQuant®
[b] Water samples consisted of 50 L filtered in a Moore swab. Each soil sample consisted of 1 k soil.
[c] Water counts reported in log CFU/ 50 L. Soil counts reported in log CFU/g.

Table 11. Counts of *Salmonella* on various environmental surface samples collected from 3 onion packing plants in the Winter Garden area of Texas

Type of surface[b]	Positive sample	Sample code	Log CFU *Salmonella*/cm^2	Means ± STDEV
Walls	1	WALL X7	6.1	1.9 ± 1.4A[c]
	2	WALL X1	2.2	
	3	WALL Y6	-3.4	
	4	WALL Y7	0.9	
	5	WALL X5A	0.6	
	6	WALL X2A	5.1	
Fans	1	FAN 4Y	3.6	2.8 ± 0.7A
	2	FAN 3Y	1.4	
	3	FAN 1Y	1.7	
	4	FAN 5Y	4.4	
Floors	1	FLOOR Y2	0.9	2.4 ± 1.0A
	2	FLOOR Y3	0.5	
	3	FLOOR Y4	4.4	
	4	FLOOR Y7	3.8	
Stair steps	1	STAIR Y6	1.4	3.2 ± 1.8A
	2	STAIR Y5	4.9	

[a] Both quantitative and qualitative tests were conducted using Bax SalQuant®.
[b] All samples represent 650 cm^2 surface.
[c] Means followed by different letters are significantly different ($P \leq 0.05$).

Table 12. Least square means for *Enterobacteriaceae* counts on various surfaces of the environment at 3 onion packing plants

Plant	Surface	Log CFU/cm^{2} [a]
A	External wall of graders	1.8 ± 0.5 A[b]
	Fans	2.5 ± 0.5 A,B
	Floors	3.1 ± 0.5 A,B
	Stair steps	2.7 ± 0.5A,B
	Walls	2.2 ± 0.5A,B
B	External wall of graders	1.1 ± 0.5 A,B
	Fans	0.8 ± 0.5 A,B
	Floors	2.2 ± 0.5A,B
	Stair steps	2.5 ± 0.5A,B
	Walls	1.4 ± 0.5B
C	External wall of graders	3.0 ± 0.6B
	Fans	1.1 ± 0.6B
	Floors	4.5 ± 0.6B
	Stair steps	2.8 ± 0.6B
	Walls	1.9 ± 0.6B

[a] Least squares means ± SE of *Enterobacteriaceae* concentration on individual packinghouse surfaces in each Plant.
[b] Means not connected by the same letter are significantly different per Student's t test ($P < 0.05$).

Table 13. Roughness of inner and outer layers of 3 onion varieties, measured using atomic force microscopy

Variety	Layer	Rq nm (root mean roughness)	Ra nm (average roughness)
White	outer	319	263
	outer	674	538
Yellow	outer	273	237
Red	outer	140	114
	outer	119	102
White	inner	35.7	28.1
Yellow	inner	8.47	7.03
	inner	8.21	6.37
	inner	9.4	7.74
Red	inner	12.7	10.4

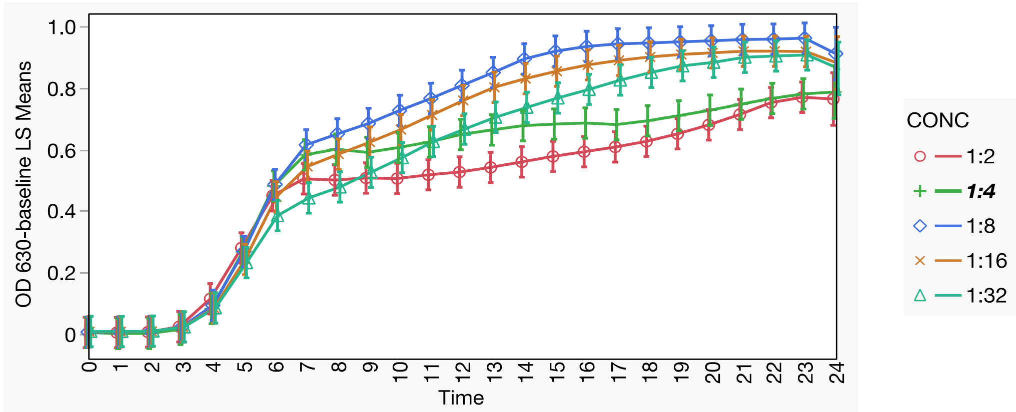

Figure 1. Growth of *Salmonella* in yellow onion extract at various concentrations.

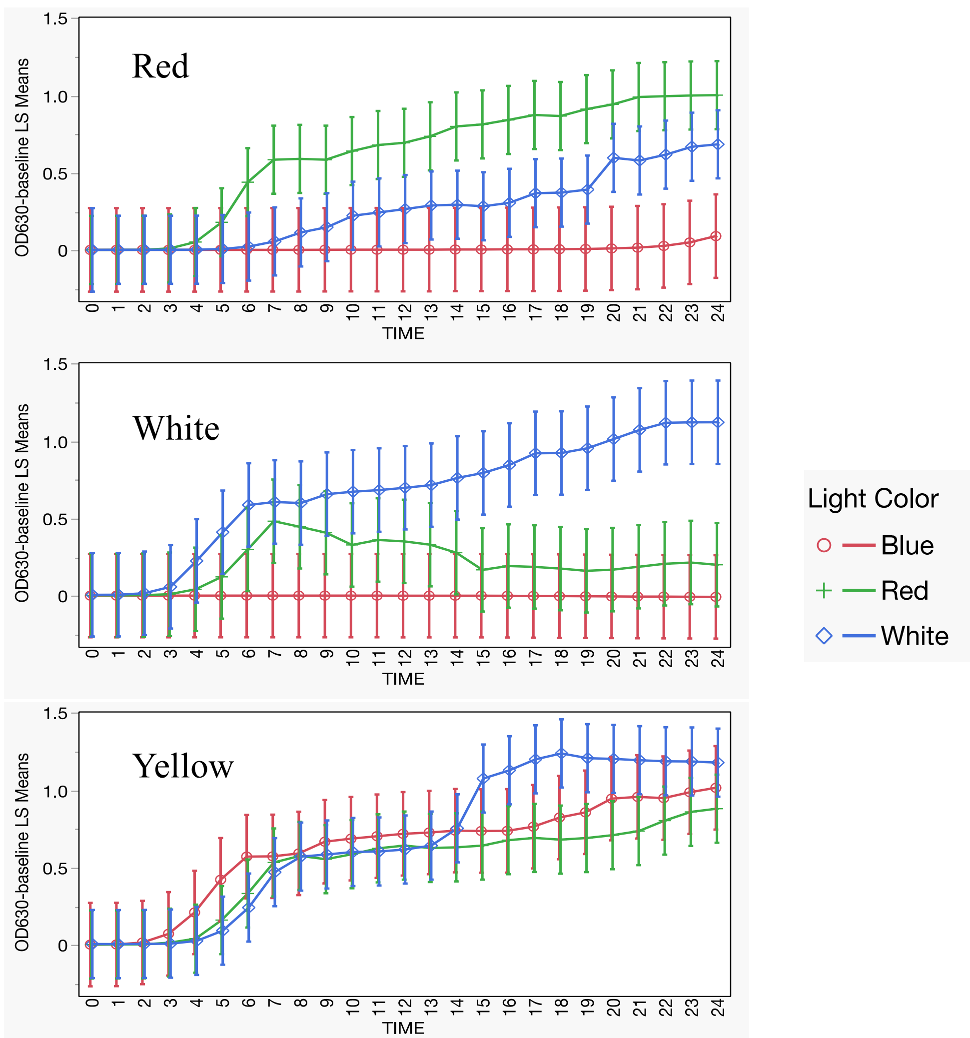

Figure 2. Survival and growth of *Salmonella* in extracts of 3 varieties of onion grown under different light colors.

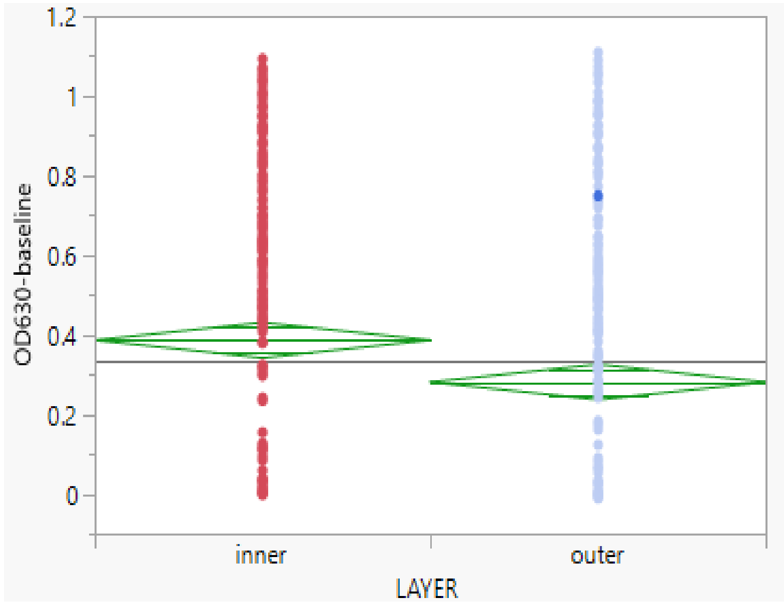

Figure 3. Effect of layer type on the growth of *Salmonella* in genotype 1104.

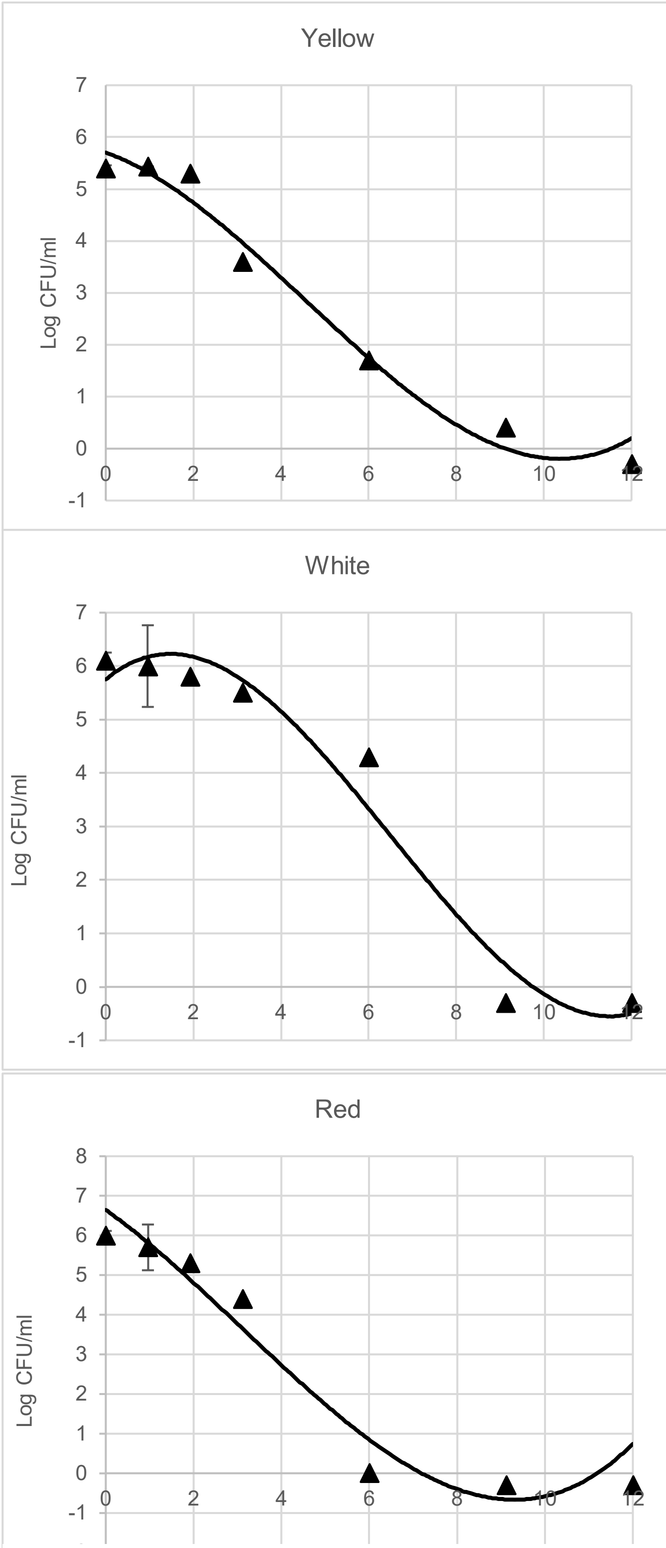

Figure 4. Survival of *Salmonella* in crude onion juices as affected by the onion variety. Regression line represents the Baranyi model using DMFit in Combase. Triangles represent experimental data.

Lag/time to shoulder.

1.19 h

5.36 h

2.12 h

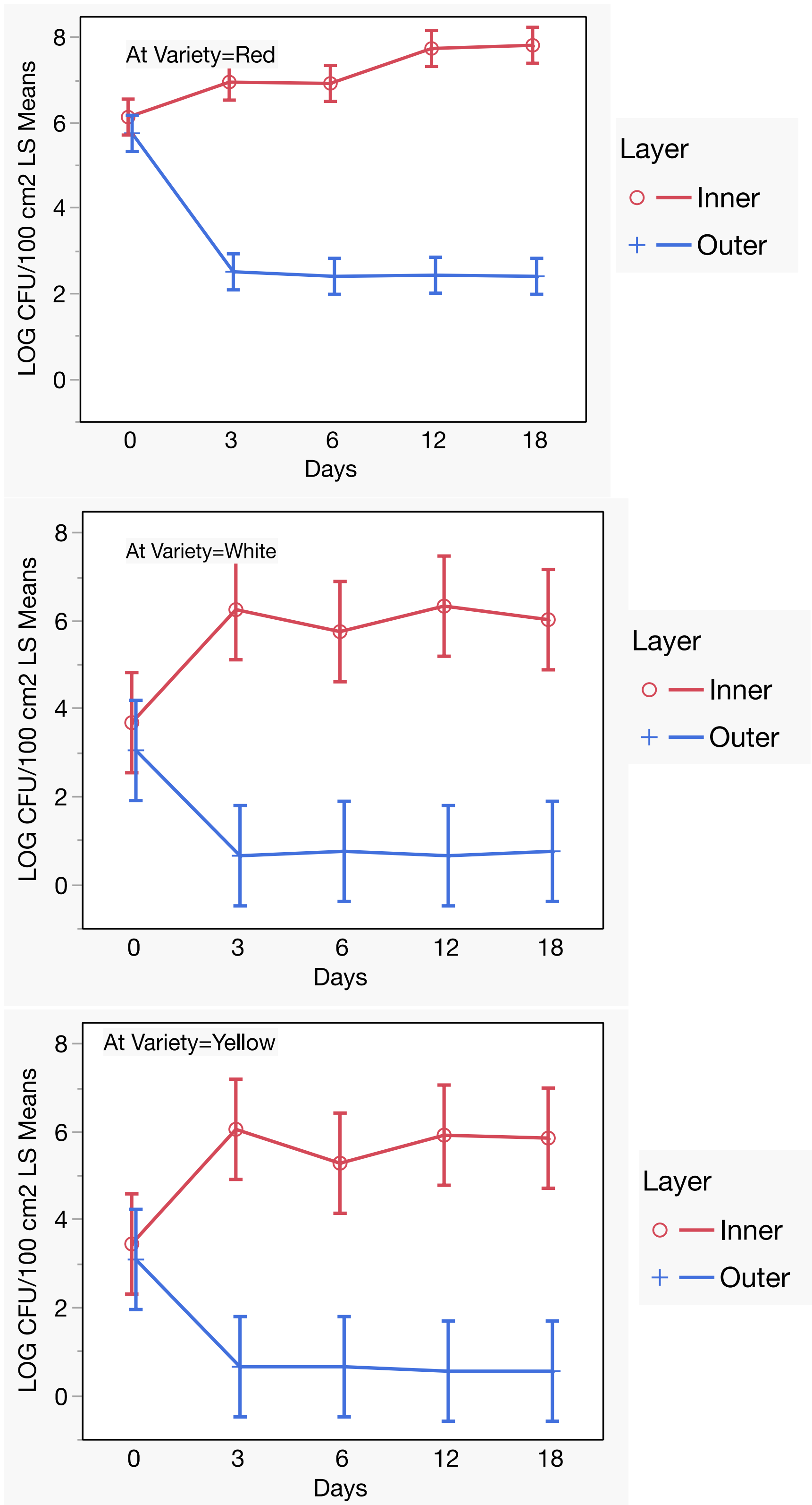

Figure 5. Survival and growth on *Salmonella* Newport on outer and inner scalers of red, white and yellow onion bulbs during storage at room temperature.

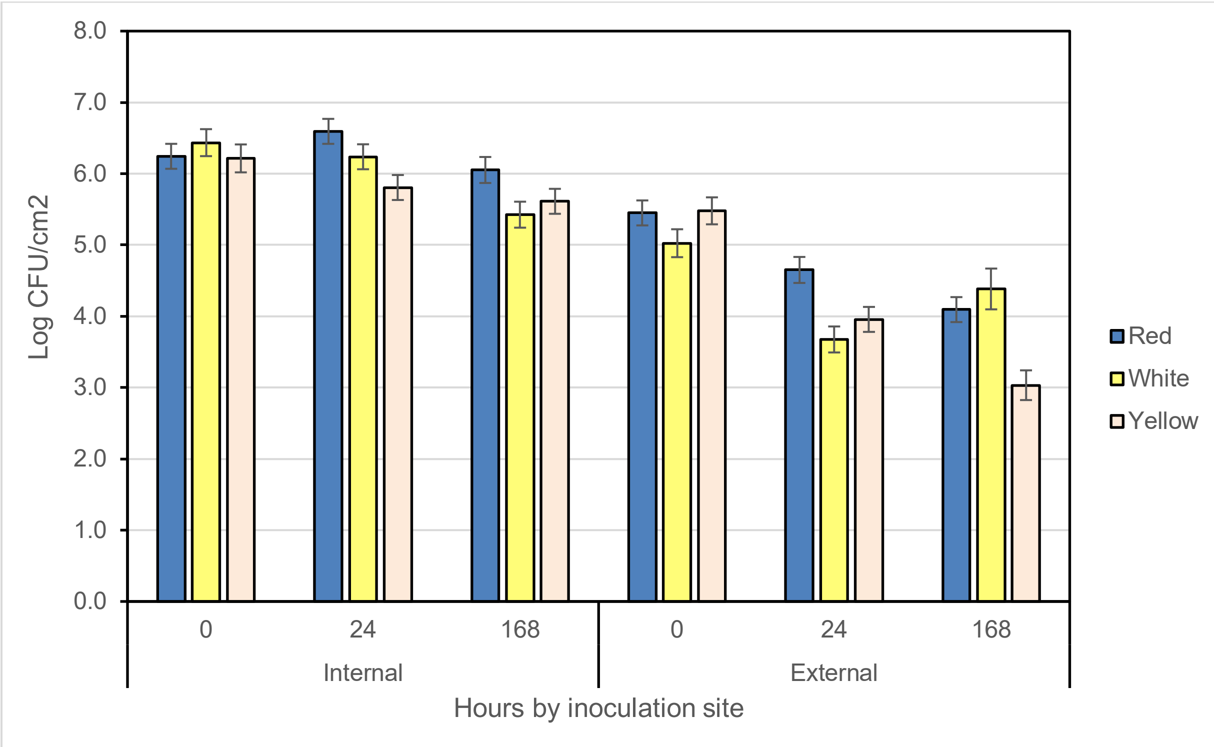

Figure 6. Counts (LSMeans) of fluorescent *Salmonella* Typhimurium inoculated internally and externally in red, white and yellow pot onions.

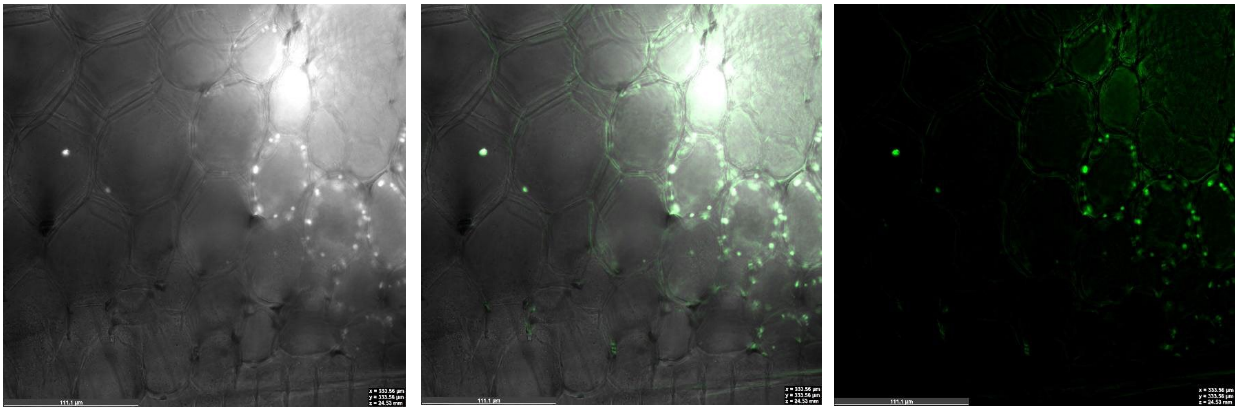

Figure 7. Bacterial internalization of GFP-labeled Salmonella Typhimurium ATCC 14028GFP on white onion tissue, 120 hours post-inoculation through damaged leaves, visualized under 40x magnification. The image on the right was taken with the GFP filter (Excitation wavelength:450-490 nm, Emission wavelength: 500-550 nm), the image on the left corresponds to a Bright Field image, and the middle image is a combination of both. Groups of GFP-labeled Salmonella Typhimurium cells were observed around the protoplasm on cells. Samples were observed and photographed under a Leica DM6 B microscope equipped with the LASX imaging system.

All samples were observed and photographed under a Leica DM6 B microscope equipped with the LASX imaging system. For the fluorescent images the excitation wavelength was 450-490 nm, and the emission wavelength was 500-550 nm. The other images correspond to Bright Field imaging.

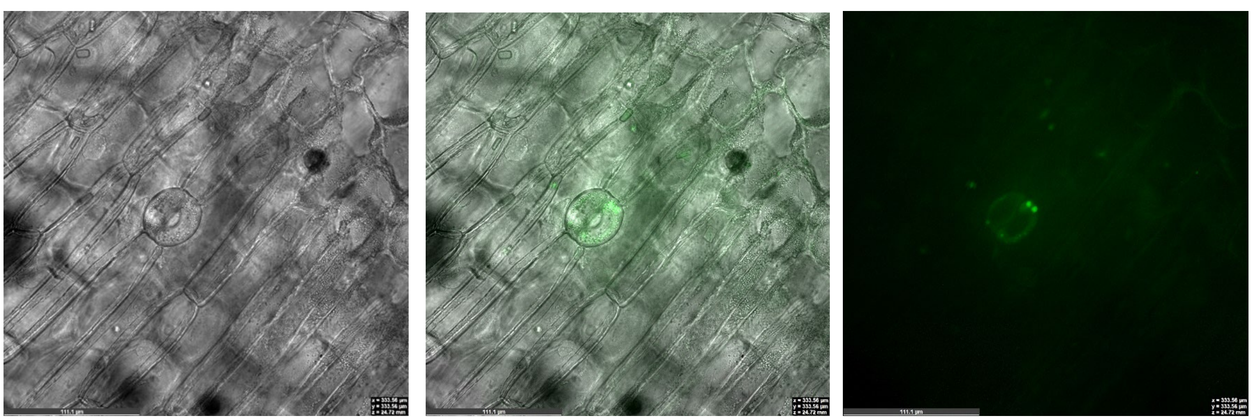

Figure 8. Bacterial internalization of GFP-labeled Salmonella Typhimurium ATCC 14028GFP on white onion tissue, 72 hours post-inoculation through damaged roots, visualized under 40x magnification. The image on the right was taken with the GFP filter (Excitation wavelength:450-490 nm, Emission wavelength: 500-550 nm), the image on the left corresponds to a Bright Field image, and the middle image is a combination of both. Cells of GFP-labeled Salmonella Typhimurium were observed near the stomata. Samples were observed and photographed under a Leica DM6 B microscope equipped with the LASX imaging system.

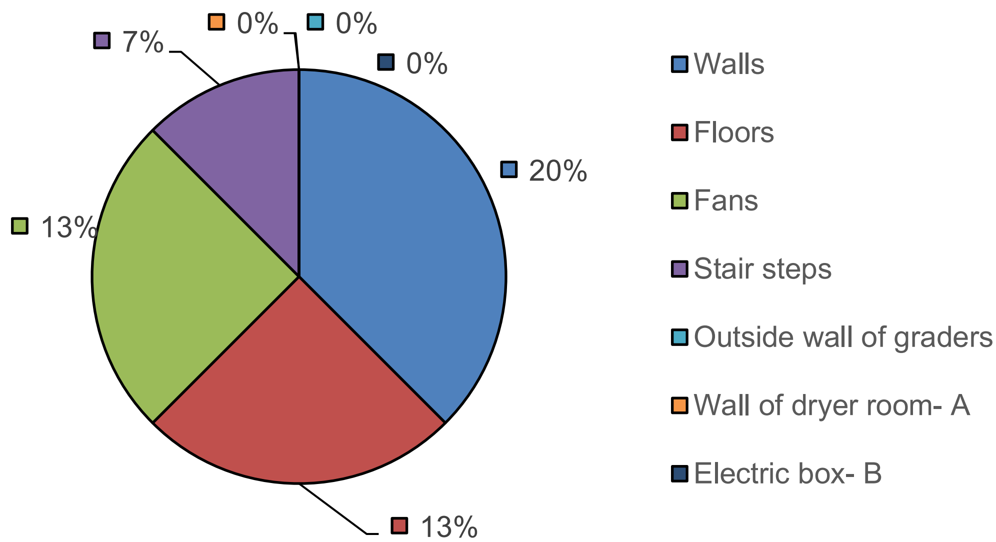

Figure 9. Percentages of isolation of *Salmonella* from diverse non-food contact surfaces collected at onion packing plants in the Uvalde-Winter Garden area in Texas. N = 154

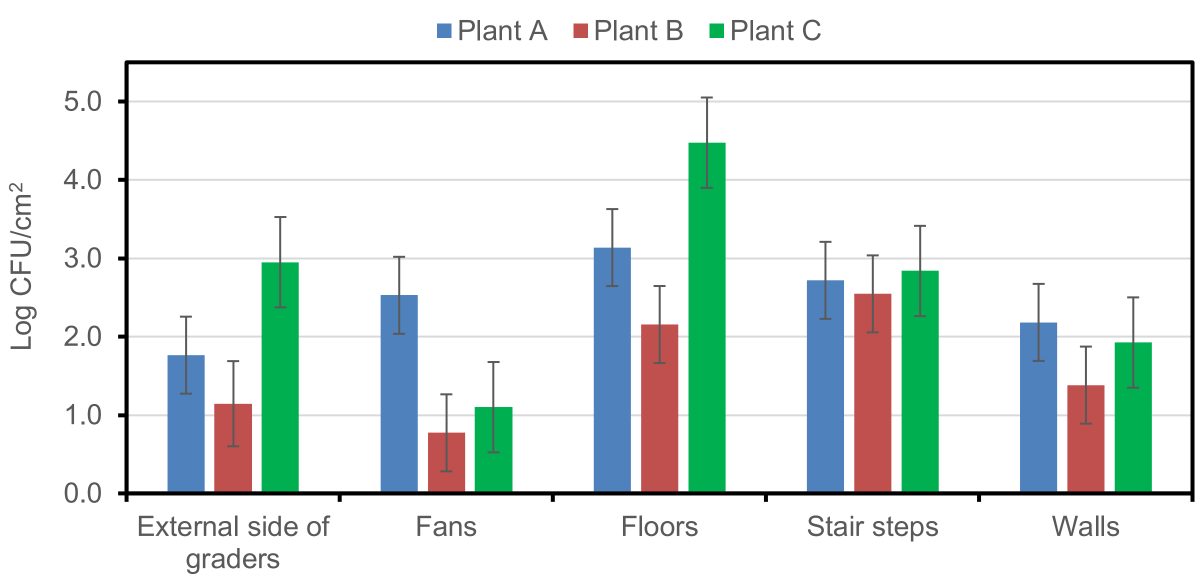

Figure 10. Counts of *Enterobacteriaceae* on surface samples collected from 3 onion packing plants in the Uvalde-Winter Garden area, Texas. Samples were collected in two separate sampling events during the 2022-2023 onion season.

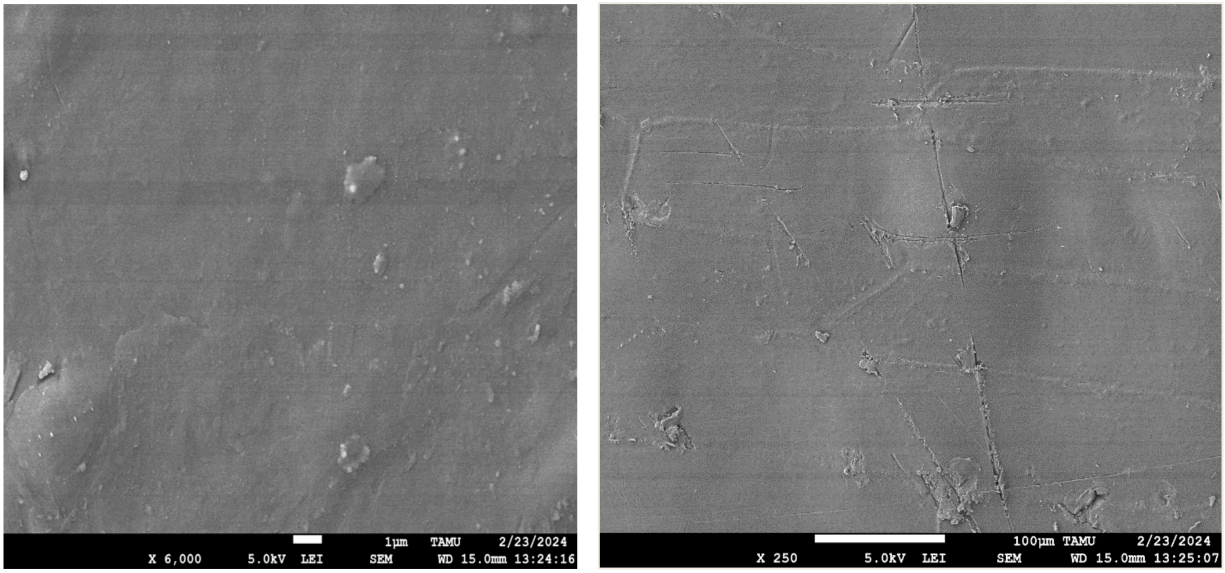

Figure 11. SEM images of red onion cultivar outer layer at 6000x and 250x magnification.

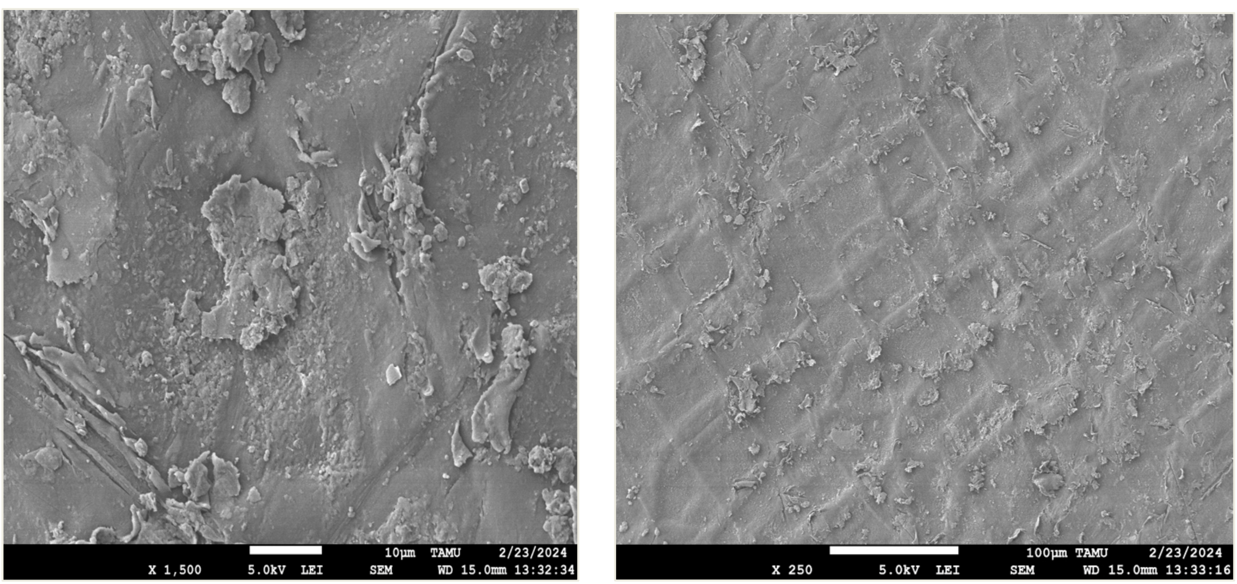

Figure 12. SEM images of white onion cultivar outer layer at 1500x and 250x magnification.

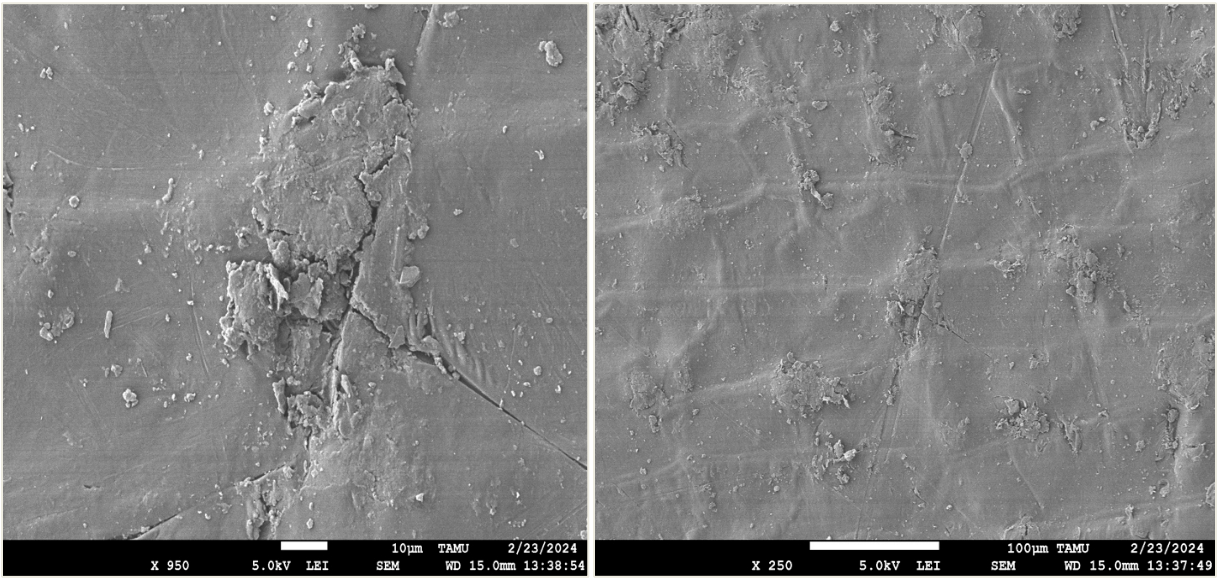

Figure 13. SEM images of yellow onion cultivar outer layer at 950x and 250x magnification.